Express Mail No.:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hong Jin *et al.* | Confirmation No.: 8169

Application No.: 09/724,388 | Group Art Unit: 1648

Filed: November 28, 2000 | Examiner: Lucas, Zachariah

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES | Attorney Docket No.: 7682-051-999

DECLARATION OF DAVID K. CLARKE
<u>UNDER 37 C.F.R. § 1.131</u>

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, DAVID K. CLARKE, who resides at 3205 Whispering Hills, Chester, New York 10918, do declare that:

1. I am a co-inventor of the invention described in the above-identified application and as claimed in pending claims 7 to 21 (annexed hereto as Exhibit A1).

2. The above-identified application and pending claims relate to genetically manipulated, infectious, replication-competent, non-segmented, negative-strand RNA viruses wherein the viral genome has been modified, *e.g.*, the genome contains deletions or insertions. The invention and pending claims encompass genetically manipulated paramyxoviruses in which the viral genome has been modified to result in a packaged infectious virion with an attenuated phenotype, *i.e.*, the virus is able to go through only one or a few rounds of replication in the host. The invention encompasses genetically manipulated RSV, vaccines comprising genetically manipulated RSV and methods for generating genetically manipulated RSV. The application claims priority to a series of

applications including earlier filed application no. 09/161,122 filed September 25, 1998, which, in turn, is a continuation-in-part of our earlier application no. 08/316,439, filed September 30, 1994 (Grand-Parent Application, attached hereto as Exhibit A2, now U.S. Patent No. 5,840,520, issued November 24, 1998).

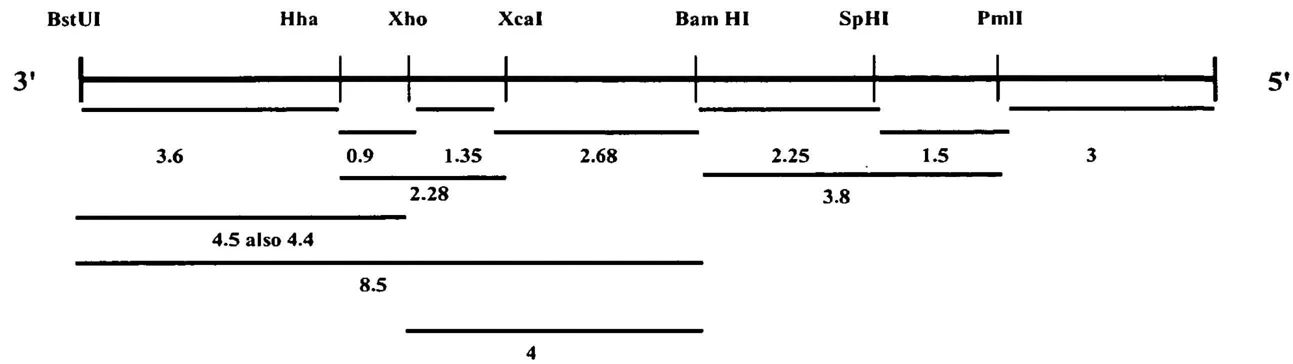
3. Annexed hereto as Exhibit B is a copy of a page from my laboratory notebook describing a research plan to genetically manipulate the genome of RSV to result in a virion with an attenuated phenotype. Such genetic manipulations include deletions and insertions of sequences in the viral genome. Although the date on the copy has been redacted, the notebook page is dated prior to January 10, 1994. Thus, demonstrating that prior to January 10, 1994, I had conceived a genetically manipulated, replication-competent, infectious, non-segmented negative strand RNA virus having an attenuated phenotype as described in the Grand Parent application and described and claimed in the instant application.

4. Annexed hereto as Exhibits C through E are copies of pages from my laboratory notebooks that demonstrate that I or person(s) acting under my direction and supervision worked with diligence from the conception of the invention up to the filing date of the Grand Parent application to reduce the claimed invention to practice. This is evidenced by the facts described below and by the attached exhibits.

5. Exhibits C to E are true copies of my laboratory notebooks that describe experiments relating to, among others, the generation of a full length cDNA encoding the genome of RSV. The pages of the laboratory notebooks are numbered consecutively for ease of reference. The references to pages in the laboratory notebooks are

to the production numbers stamped at the bottom right corner of the pages in the notebooks. The dates in the laboratory notebooks have been redacted. Further, references to events that would lead to the date of the entry into the laboratory notebook have also been redacted. The laboratory notebooks document experiments that were conducted during the time period from briefly before the date of the conception document until the filing date of the Grand Parent application.

6. In particular, the laboratory notebooks document the isolation of genomic RSV RNA, RT-PCR amplification of fragments of the RSV genome, optimization of growth cultures of RSV, the subcloning of the RT-PCR fragments of the RSV genome in different cloning vectors and the ligation of the different RT-PCR fragments to obtain a cDNA encoding the full length genome of RSV. The fragments of the RSV genome that are mentioned in the laboratory notebooks are set forth in the following genomic restriction map of RSV. Restriction enzyme sites are shown on above the line representing the genome and the numbers below the line refer to the approximate sizes of the fragments in kilobasepairs. In this declaration, DNA fragments are referred to by the name of the flanking restriction sites followed by the approximate length of the fragment. For example, the first fragment at the 3' end of the virus is designated BstU/Hha-3.5. Synonyms of the DNA fragments are listed in the respective paragraphs that describe the work relating to the different DNA fragments. The map below also shows how the different DNA fragments were to be assembled to obtain the full length RSV cDNA.



Genomic Restriction Map of RSV

7. The following paragraphs explain the type of experiments to obtain the different DNA fragments shown in the diagram above and the experiments to assemble these fragments into a full-length RSV cDNA; references in the following paragraphs are to production numbers in Exhibits C to E. In general, the entries in connection with obtaining the DNA fragments relate to RT-PCR amplification of the fragment, purification of the amplified DNA material, ligation into a cloning vector, transfection into bacteria, preparation of plasmid DNA from the transfected bacteria, and restriction analysis of the plasmid DNA.

7a. Work relating to the growth of RSV, infection of different cell types with RSV, the harvest and purification of virus, and the purification of genomic RNA of RSV are described at production nos. 47-65, 67-70, 72-79, 81, 95, 105, 110, 113, 117, 121-125, 127, 133, 137-144, 146, 148, 150-151, 153, 155, 169, 176-177, 188, 193, 212, 249, 269, 322, and 353-354 of my laboratory notebooks. These entries also relate to obtaining RS virus stocks from different sources, the optimization of growth conditions for RSV, testing of different cell lines for virus replication, optimization of virus preparation, and the purification of genomic RNA from the virus.

7b. Work relating to the performance of RT-PCR to obtain DNA fragments that encode parts of the viral genome are described at production nos. 65-66, 83-84, 87, 89, 90, 157, 160-165, 170, 172, 173, 179-180, 205, 242, 246-247, 282, and 393-394 of my laboratory notebooks. These entries also relate to obtaining the reagents and biochemicals for conducting RT-PCR, designing of primers, designing of cloning strategies, and the optimization of the conditions for the performance of RT-PCR. Work relating to the preparation of cloning vectors for subcloning of the RT-PCR fragments is described at production nos. 115-116 (puc19),

7c. Work relating to the RT-PCR amplification and the subcloning of the BstU/Hha-3.6 fragment (also referred to as "BstU/Hha" and "3.5K" and "3.6K" and "3.7K") is described at production nos. 264, 270, 272, 274, 276-277, 279, 281, 298, 301-302, 322-323, 325, 328, 339, 343, 367, 369-371, 378, and 381-382 of my laboratory notebooks. These entries also relate to large scale plasmid preparation of the cloned fragment.

7d. Work relating to the RT-PCR amplification and the subcloning of the Hha/Xho-0.9 fragment (also referred to as "0.9K" and "H/Xho" and "Hha/Xho" and "clone 8") is described at production nos. 327-331, 336-337, 345-350, 352, 361-362, 366-368, 371, 376, 378-380, 382, 386-387, and 392 of my laboratory notebooks. These entries also relate to the development of alternative cloning strategies, and the evaluation of restriction analysis.

7e. Work relating to the RT-PCR amplification and the subcloning of the Xho/Xca-1.35 fragment (also referred to as "1.3K" (370) and "Xho/Xca" and "clone 2") is described at production nos. 295-298, 299, 301, 331, 336-337, 345-350, 352, 358-368, 370, 374, 377-380, 382, 386-387, 393, 399, 423, 426-427, 429-432, 434, 459-461, 465-467, 471-

473, 475-476, and 481-487 of my laboratory notebooks. These entries also relate to the development of alternative cloning strategies, and the evaluation of restriction analysis.

7f. Work relating to the RT-PCR amplification and the subcloning of the Xca/BamHI-2.68 fragment (also referred to as "Xca/Bam" and "Bam/Xca" and "X/B" and "2.6K" and "2.7K") is described at production nos. 261-262, 264, 268, 270-271, 274-275, 306, 322, 343, 369-370, 373-374, and 377-378 of my laboratory notebooks.

7g. Work relating to the RT-PCR amplification and the subcloning of the BamHI/SpHI-**2.25** fragment (also referred to as "BamHI/SphI" and "Bam/Sph" and "2.25Kb" or "2.25K") is described at production nos. 258-260, 268, 270-271, 274-275, 305-306, 343, 354-357, 407-411, 433, 457, 459, 461-464, 472, 475, 480, 484, and 486-490 of my laboratory notebooks. These entries also relate to restriction mapping of the BamHI/SpHI-2.25 fragment.

7h. Work relating to the RT-PCR amplification and the subcloning of the SpHI/PmlI-**1.5** fragment (also referred to as "1.5K") is described at production nos. 250-254, 256-257, 259-260, 263, 266-267, 305, 343, 354-357, 409-410, 412, 418-420, 422-423, 425-434, 457, 461-464, 486, and 488 of my laboratory notebooks. These entries also relate to restriction mapping of the SpHI/PmlI-1.5 fragment.

7i. Work relating to the RT-PCR amplification and the subcloning of the PmlI/5'end-**3** fragment (also referred to as "3K" and "3K sure") is described at production nos. 179-180, 189-190, 193-196, 198, 298, 301-303, 309, 311, 316, 343, 407-409, 411-412,

418-424, 433, and 457 of my laboratory notebooks. These entries also relate to large scale plasmid preparation and the restriction analysis of the plasmid DNA.

7j. Work relating to the RT-PCR amplification and subcloning of the BamHI/PmlI-3.8 fragment (also referred to as "3.8Kb") is described at production nos. 181, 185,194, 196, 197, 201-202, 204, 207-210, 214-216, 240-242, and 243 of my laboratory notebooks. These entries also relate to designing of primers to amplify the BamHI/PmlI-3.7 fragment.

7k. Work relating to the RT-PCR amplification and subcloning of the HhaI/XcaI-2.28 fragment (also referred to "2.3k" and "Hha/Xca" and "H/Xca" and "Sure 2.3" and "H/X") is described at production nos. 261-262, 268, 275-281, 285, 287-294, 299-300, 305, 307, 309, 311-321, 324, 327-330, 332, 334-336, 337, 339-345, 372-374, and 388-391 of my laboratory notebooks. These entries also relate to designing of primers to amplify the HhaI/XcaI-2.28 fragment and the development of alternative cloning strategies, such as the use of different competent bacterial cells for the propagation of the plasmid DNA.

7l. Work relating to the RT-PCR amplification and subcloning of the BstUI/XhoI-4.5 fragment (this fragment is also referred to as "4.4K") is described at production nos. 325-326, 414-417, and 421 of my laboratory notebooks.

7m. Work relating to the RT-PCR amplification and subcloning of the Xho/BamHI-4 fragment (also referred to as "4K" and "Xho/Bam)is described at production nos. 205-207, 209-211, 214, 379, and 383-384 of my laboratory notebooks.

7n. Work relating to the generation of the leader and trailer regions of the RSV is described at production nos. 92-94, 113-114, 127, 129-136, 138, 139, 283-284, and 405 of my laboratory notebooks.

7o. Work relating to the ligation of the BstU/Hha-3.6 fragment, the HhaI/XcaI-2.28, and the Xca/BamHI-2.68 fragment to result in BstUI/BamHI-8.5 are described at production nos. 347-352 of my laboratory notebooks.

7p. Work relating to the ligation of the BstU/Hha-3.6 fragment and the Hha/Xho-0.9 fragment to obtain a the BstU/Xho-4.4 fragment (also referred to as "4.4k") is described at production nos. 391-392, 395-398, 400-401, 403, 406, 413, 469, 471, 473, 476-482, 484-485, 489-490 of my laboratory notebooks.

7q. Work relating to the ligation of the BstU/Xho-4.4 fragment and the Xho/Xca-1.35 fragment is described at production nos. 474 and 485 of my laboratory notebooks.

7r. Work relating to the ligation of two or three of the BamHI/SpHI-2.25 fragment, the SpHI/PmlI-1.5 fragment, and the PmlI/5'end-3 fragment to each other is described at production nos. 425, 458, 465-466, 468-473, 475, and 491 of my laboratory notebooks.

8. The fragments that are described in paragraphs 7a to 7s were later used to a assemble full-length cDNA that encodes the genome of an RSV, which was used to rescue an infectious and replication-competent RSV. The full-length cDNA was genetically

manipulated to generate RSV cDNAs with deletions and insertions, which was used to rescue an infectious and replication-competent RSV with an insertion and/or deletion.

9. Exhibit F is a summary of the work described in the laboratory notebooks that are submitted herewith as Exhibits C to E. Each row in Exhibit F represents one day or, where the experiment was performed over several days, a row represents the corresponding period of time.

10. I declare that since the date of conception of the invention I was diligently working towards the reduction to practice of the invention. The only interruption of my work toward the reduction to practice in the laboratory of more than one week was during the two weeks immediately prior to filing of the Grand Parent application. However, during my absence from the laboratory, my technician, Lisa Paganini, was working on the ligation of the SphI/PmlI-1.5 kb fragment and the BamHI/SphI-2.25 kb fragments to each other in accordance with my instructions as indicated at production no. 491.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: March 24th 2004

David K Clarke
DAVID K. CLARKE

EXHIBIT A1: COPY OF PENDING CLAIMS IN U.S. APPLICATION NO. 09/724,388 AS OF MARCH 24, 2004

7. (Currently amended) A genetically manipulated, replication competent, infectious virus of the paramyxoviridae family wherein the virus genome comprises a modification wherein the modification is selected from an insertion, ~~substitution~~ or deletion.

8. (Previously presented) The genetically manipulated, infectious virus of Claim 7, the genome of which has been modified to encode a heterologous sequence.

9. (Withdrawn) The virus of Claim 7 or 8 wherein the virus is a parainfluenza virus.

10. (Previously presented) The virus of Claim 7 or 8 wherein the virus is a respiratory syncytial virus.

11. (Currently amended) A genetically manipulated, replication competent, infectious, non-segmented, negative-stranded RNA virus wherein the virus genome comprises a modification wherein the modification is selected from an insertion, ~~substitution~~ or deletion.

12. (Previously presented) A vaccine comprising the genetically manipulated viruses of Claims 7, 8, or 11 and a pharmaceutically acceptable carrier.

13. (Withdrawn) A method for rescuing a genetically manipulated non-segmented negative-stranded RNA virus comprising

introducing into a host cell expressing a heterologous RNA polymerase:

(a) one or more DNA molecules encoding the virus N, P and L proteins operably linked to a polymerase binding site;

(b) a DNA molecule comprising the cDNA of the non-segmented negative stranded RNA virus wherein the cDNA encodes the entire genome of the virus or is modified by the incorporation of a mutation or a heterologous sequence, and wherein the cDNA is transcribed by a heterologous RNA polymerase; and

isolating the virus produced by the cell.

14. (Withdrawn) The method of Claim 13 wherein the virus is from the paramyxoviridae family.

15. (Withdrawn) The method of Claim 14 wherein the virus is parainfluenza virus.

16. (Withdrawn) The method of Claim 14 wherein the virus is a respiratory syncytial virus.

17. (New) The virus of claim 8, wherein the heterologous sequence is a non-RSV sequence.

18. (New) A genetically manipulated, replication-competent, infectious non-segmented, negative strand RNA virus wherein the virus genome comprises a modification wherein the modification is selected from an insertion, substitution, or deletion of an open reading frame encoding a viral gene product.

19. (New) The virus of claim 18, wherein the virus is from the paramyxoviridae family.

20. (New) The virus of claim 18, wherein the virus is RSV.

21. (New) The virus of claim 18, wherein the open reading frame is the M-2 open reading frame.

US005840520A

United States Patent [19]

Clarke et al.

[11] **Patent Number: 5,840,520**

[45] **Date of Patent: *Nov. 24, 1998**

[54] **RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS**

[75] Inventors: **David Kirkwood Clarke**, Pacifica, Calif.; **Peter M. Palese**, Leonia, N.J.

[73] Assignee: **Aviron**, Mountain View, Calif.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,166,057.

[21] Appl. No.: **316,439**

[22] Filed: **Sep. 30, 1994**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 190,698, Feb. 1, 1994, abandoned, which is a continuation of Ser. No. 925,061, Aug. 4, 1992, abandoned, which is a division of Ser. No. 527,237, May 22, 1990, Pat. No. 5,166,057, which is a continuation-in-part of Ser. No. 440,053, Nov. 21, 1989, abandoned, and Ser. No. 399,728, Aug. 28, 1989, abandoned.

[51] **Int. Cl.**[6] **C12P 21/06**; C12P 19/34; A61K 39/12

[52] **U.S. Cl.** **435/69.1**; 435/91; 435/235.1; 435/320.1; 424/199.1; 536/23.1

[58] **Field of Search** 435/69.1, 91, 172.3, 435/235.1, 320.1; 536/23.1; 424/199.1

[56] **References Cited**

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(List continued on next page.)

Primary Examiner—Michael P. Woodward
Assistant Examiner—Ali R. Salimi
Attorney, Agent, or Firm—Pennie & Edmonds LLP

[57] **ABSTRACT**

Recombinant negative strand virus RNA templates which may be used to express heterologous gene products and/or to construct chimeric viruses are described. Influenza viral polymerase, which was prepared depleted of viral RNA, was used to copy small RNA templates prepared from plasmid-encoded sequences. Template constructions containing only the 3' end of genomic RNA were shown to be efficiently copied, indicative that the promoter lay solely within the 15 nucleotide 3' terminus. Sequences not specific for the influenza viral termini were not copied, and, surprisingly, RNAs containing termini identical to those from plus sense cRNA were copied at low levels. The specificity for recognition of the virus-sense promoter was further defined by site-specific mutagenesis. It was also found that increased level of viral protein were required in order to catalyze both the cap-endonuclease primed and primer-free RNA synthesis from these model templates as well as from genomic length RNAs. This indicated that this reconstituted system had catalytic properties very similar to those of native viral RNPs. High levels of expression of a heterologous gene was obtained using the constructs and methods described. The system was exemplified using Influenza and respiratory syncytial virus.

9 Claims, 23 Drawing Sheets

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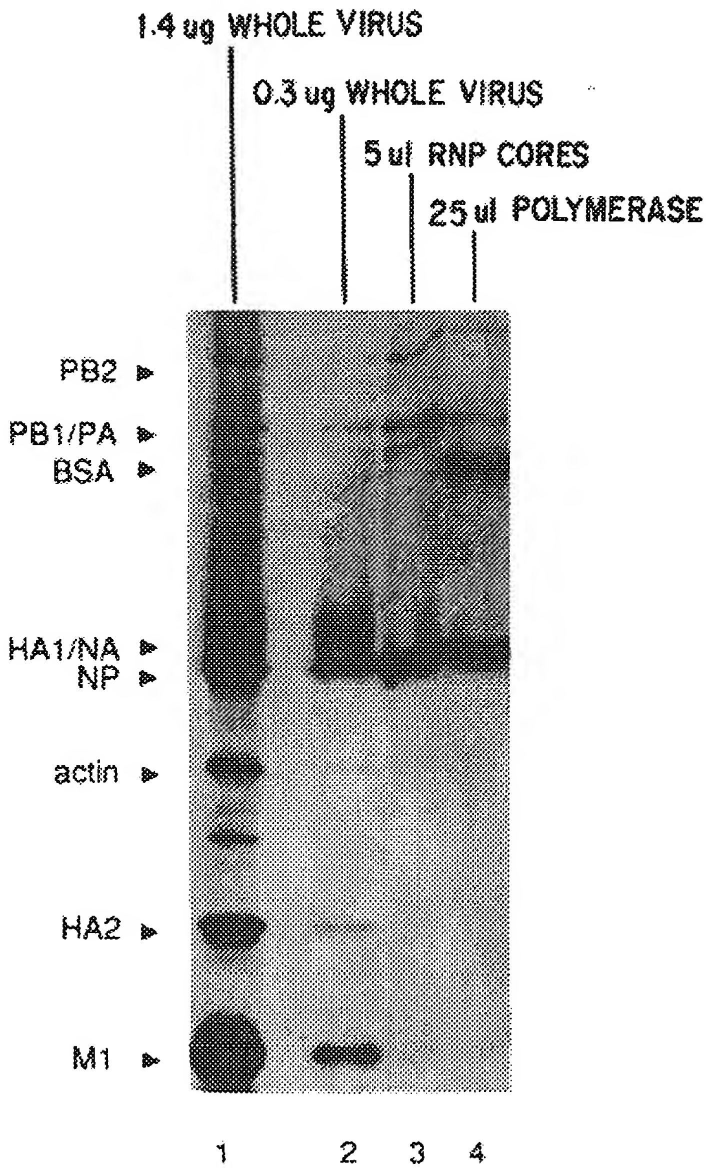

1.4 ug WHOLE VIRUS
0.3 ug WHOLE VIRUS
5 ul RNP CORES
25 ul POLYMERASE
PB2
PB1/PA
BSA
HA1/NA
NP
actin
HA2
M1
1
2
3
4

FIG. 1

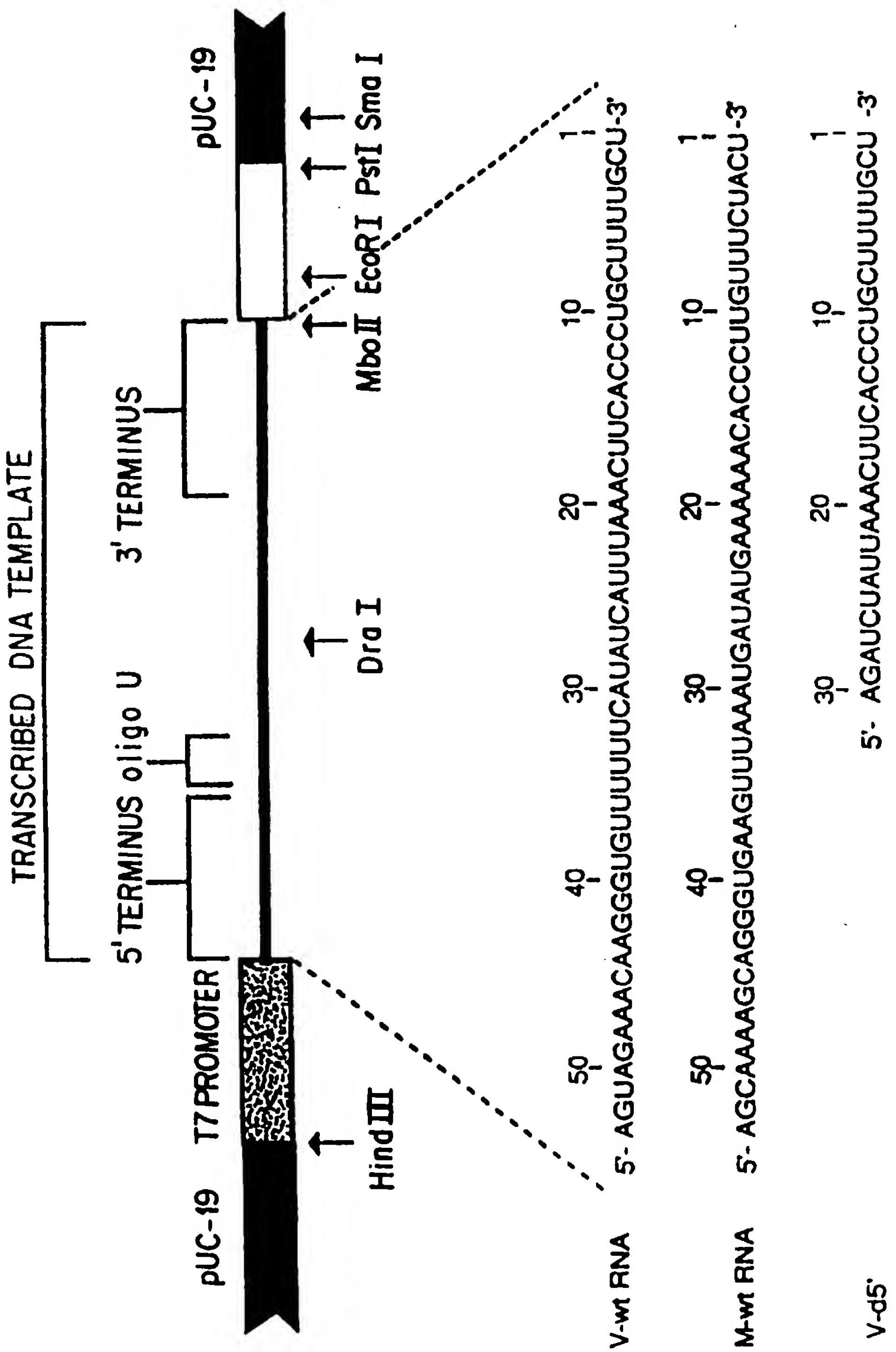

TRANSCRIBED DNA TEMPLATE
5' TERMINUS
oligo U
3' TERMINUS
pUC-19
T7 PROMOTER
pUC-19
Hind III
Dra I
Mbo II
EcoR I
Pst I
Sma I
V-wt RNA
5'- AGUAGAAACAAGGGUGUUUUUUCAUAUCAUUUAAACUUCACCCUGCUUUUGCU-3'
M-wt RNA
5'- AGCAAAAGCAGGGUGAAGUUUAAAUGAUAUGAAAAAACACCCUUGUUUCUACU-3'
V-d5'
5'- AGAUCUAUUAAACUUCACCCUGCUUUUGCU -3'
50
40
30
20
10
1

FIG. 2

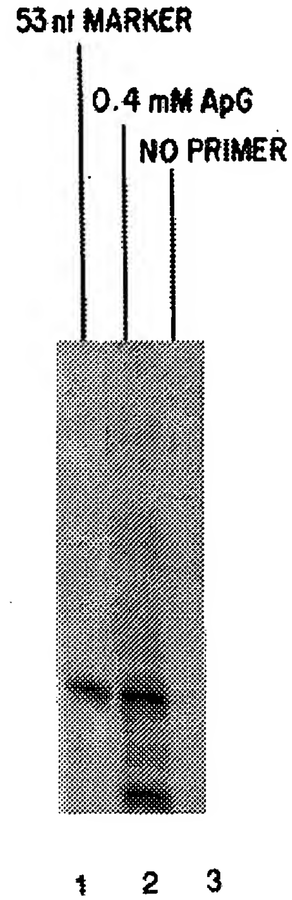


FIG. 3A

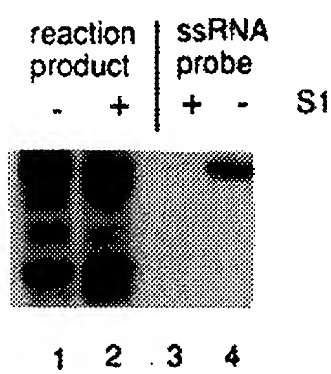


FIG. 3B

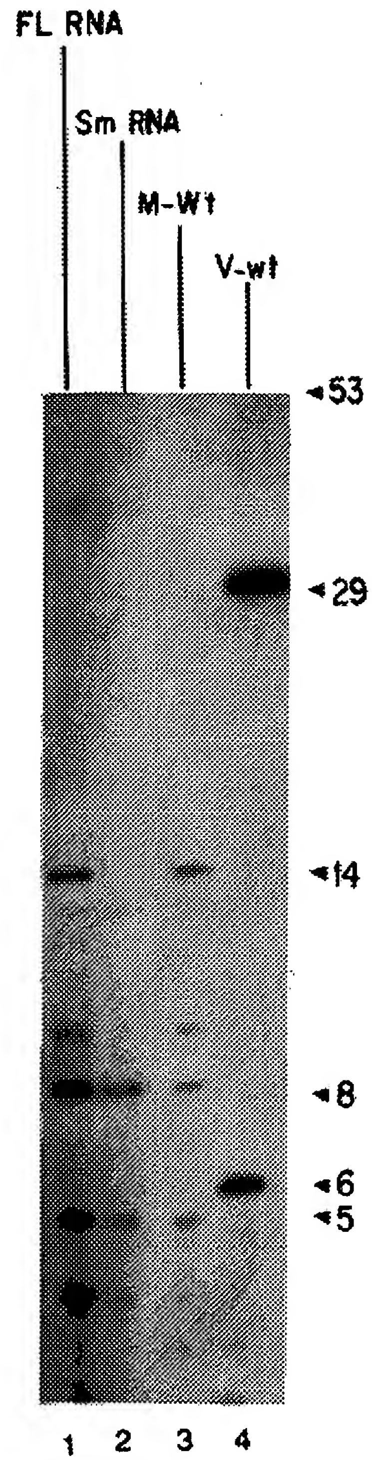


FIG. 3C

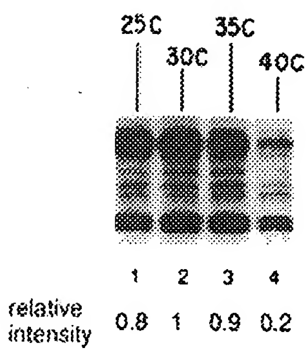


FIG. 4A

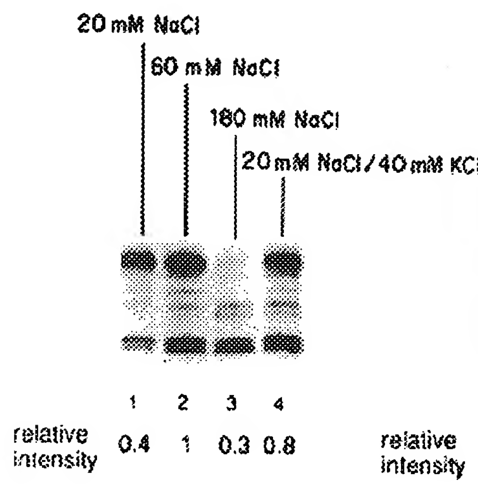


FIG. 4B

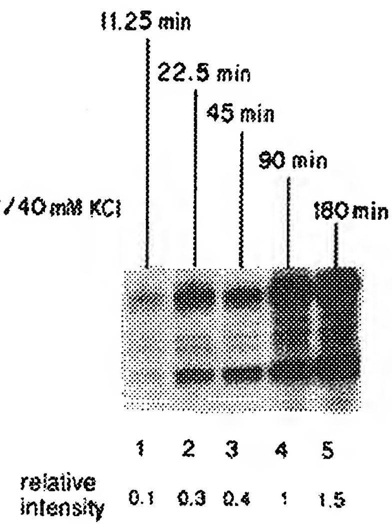


FIG. 4C

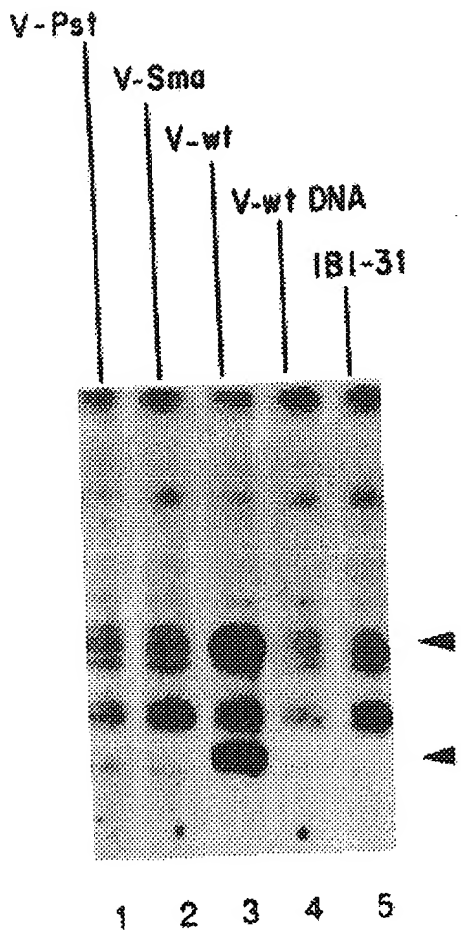

V-Pst
V-Sma
V-wt
V-wt DNA
IBI-31
1
2
3
4
5

FIG. 5A

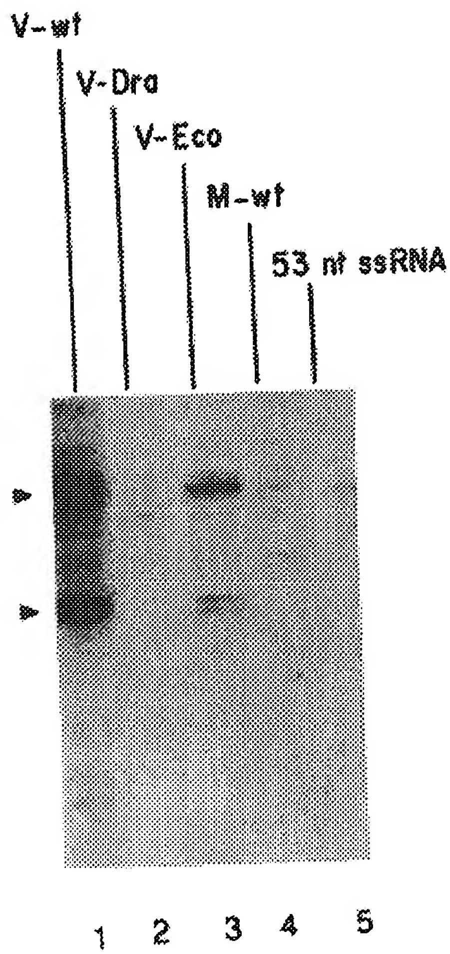

V-wt
V-Dra
V-Eco
M-wt
53 nt ssRNA
1
2
3
4
5

FIG. 5B

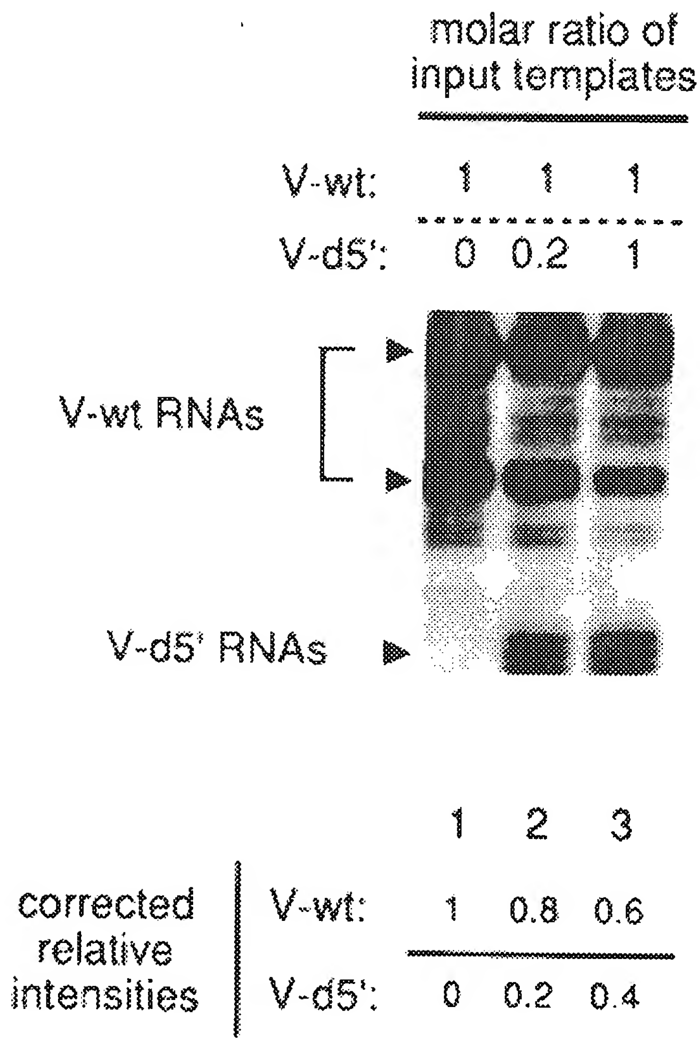

molar ratio of
input templates
V-wt: 1 1 1
V-d5': 0 0.2 1
V-wt RNAs
V-d5' RNAs
1 2 3
corrected
relative
intensities
V-wt: 1 0.8 0.6
V-d5': 0 0.2 0.4

FIG. 6

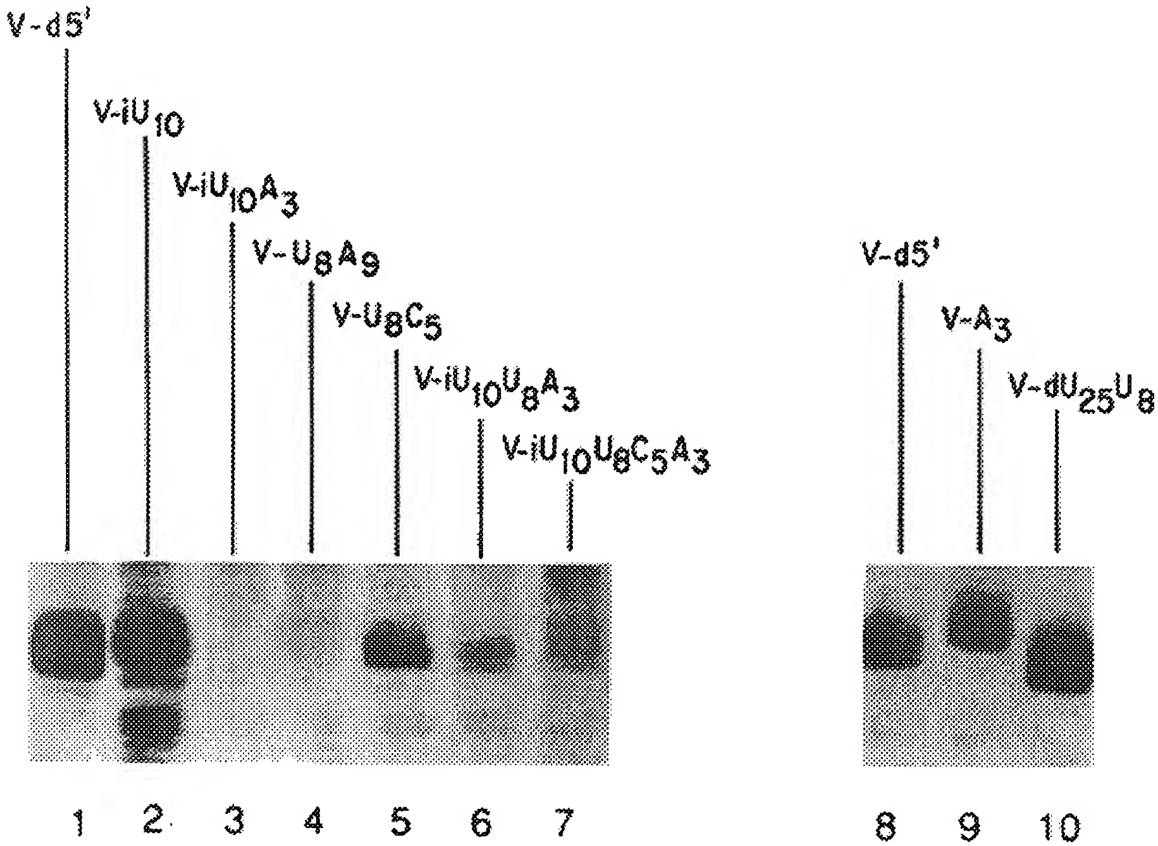
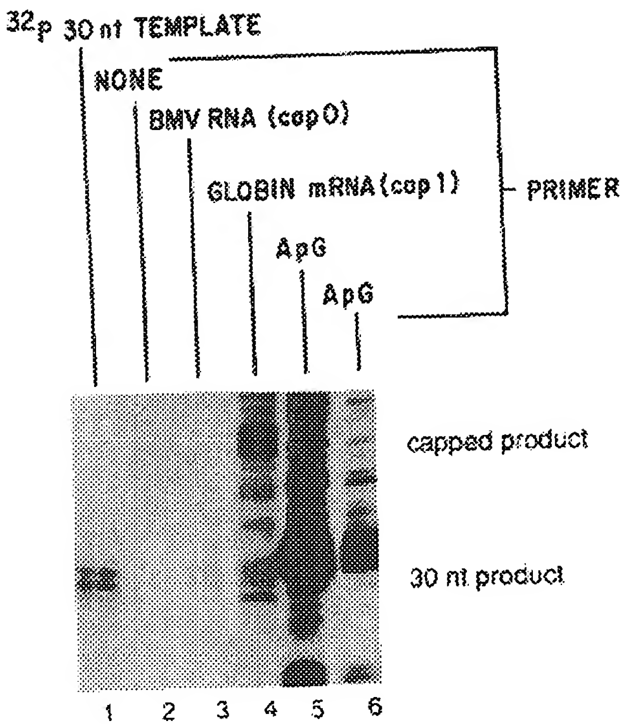
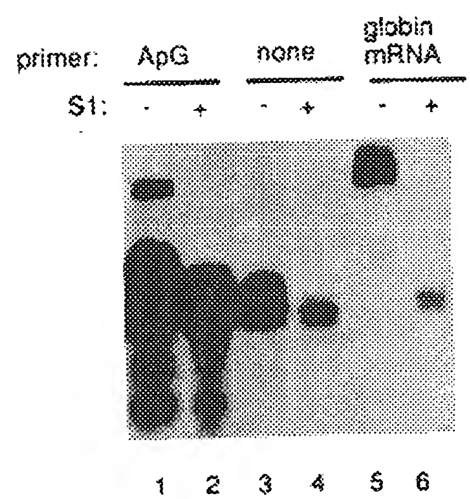

V-d5'
V-iU10
V-iU10A3
V-U8A9
V-U8C5
V-iU10U8A3
V-iU10U8C5A3
V-d5'
V-A3
V-dU25U8
1
2
3
4
5
6
7
8
9
10

FIG. 7



32P 30 nt TEMPLATE
NONE
BMV RNA (cap0)
GLOBIN mRNA (cap1)
PRIMER
ApG
ApG
capped product
30 nt product
1 2 3 4 5 6

FIG. 8A



primer: ApG none globin mRNA
S1: - + - + - +
1 2 3 4 5 6

FIG. 8B

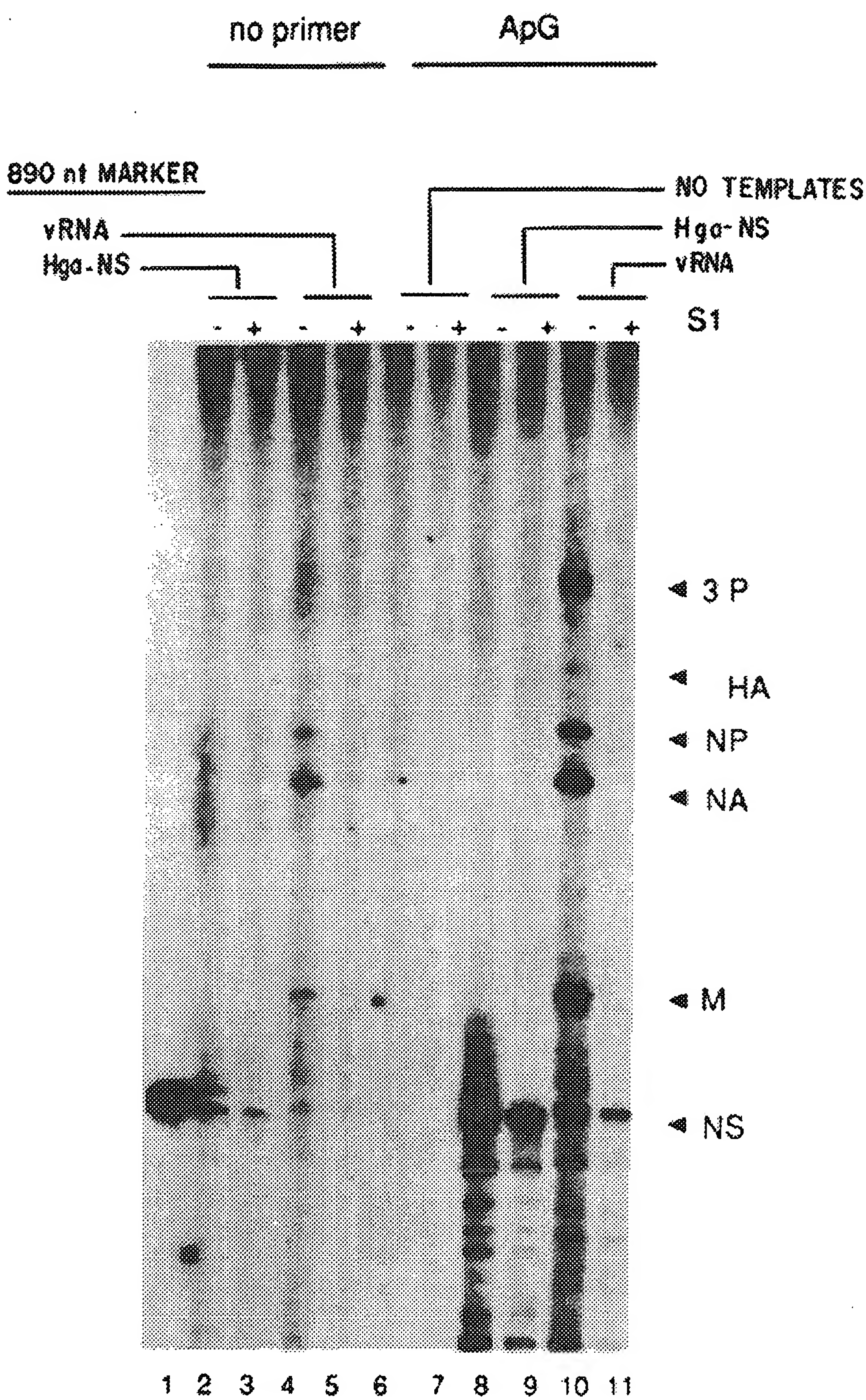
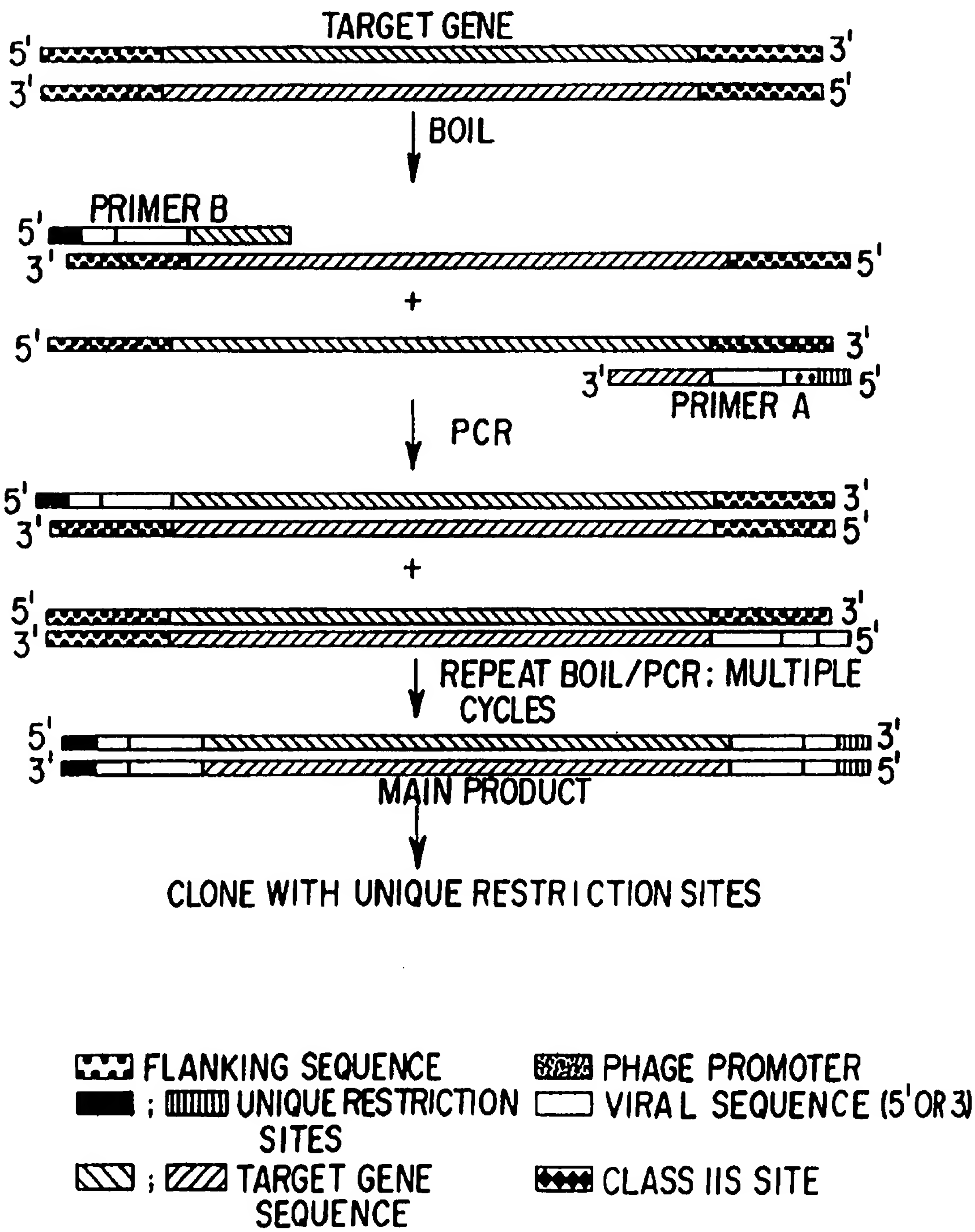

no primer
ApG
890 nt MARKER
vRNA
Hga-NS
NO TEMPLATES
Hga-NS
vRNA
S1
- + - + - + - + - +
3 P
HA
NP
NA
M
NS
1 2 3 4 5 6 7 8 9 10 11

FIG. 9

FIG. 10


TARGET GENE
5'
3'
3'
5'
BOIL
PRIMER B
5'
3'
5'
+
5'
3'
3'
5'
PRIMER A
PCR
5'
3'
3'
5'
+
5'
3'
3'
5'
REPEAT BOIL/PCR: MULTIPLE CYCLES
5'
3'
3'
5'
MAIN PRODUCT
CLONE WITH UNIQUE RESTRICTION SITES
FLANKING SEQUENCE
; UNIQUE RESTRICTION SITES
; TARGET GENE SEQUENCE
PHAGE PROMOTER
VIRAL SEQUENCE (5' OR 3)
CLASS IIS SITE

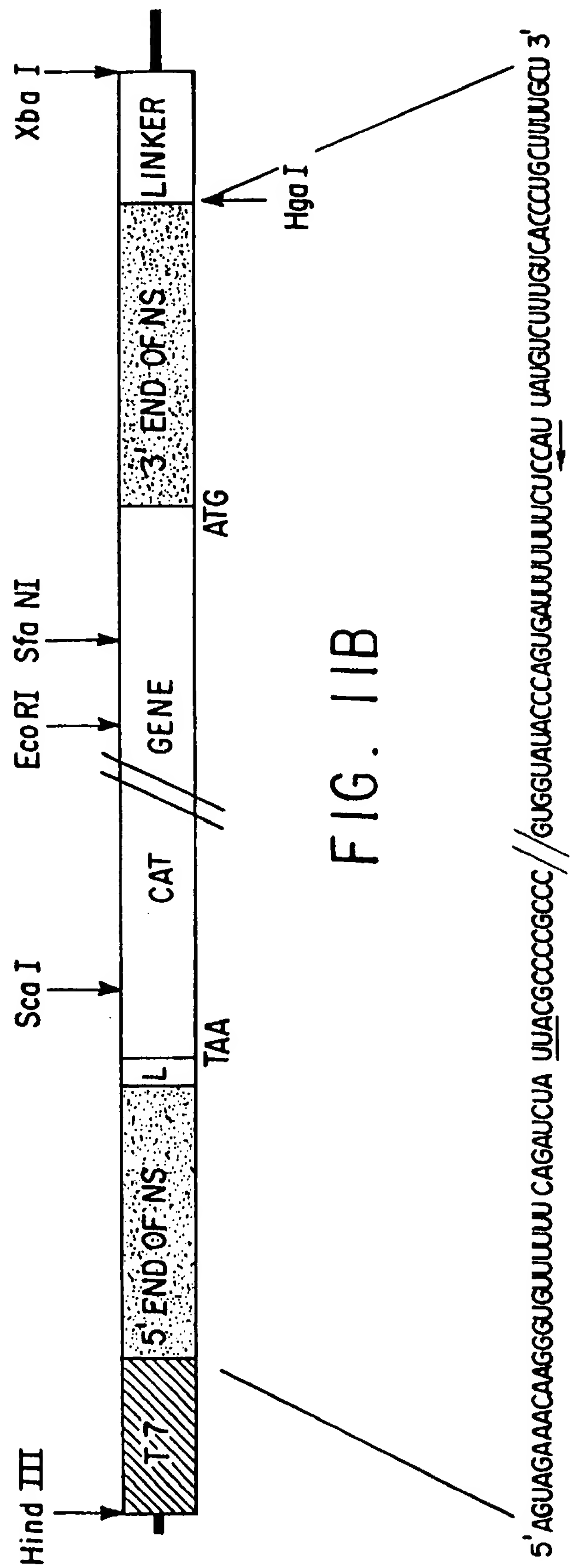
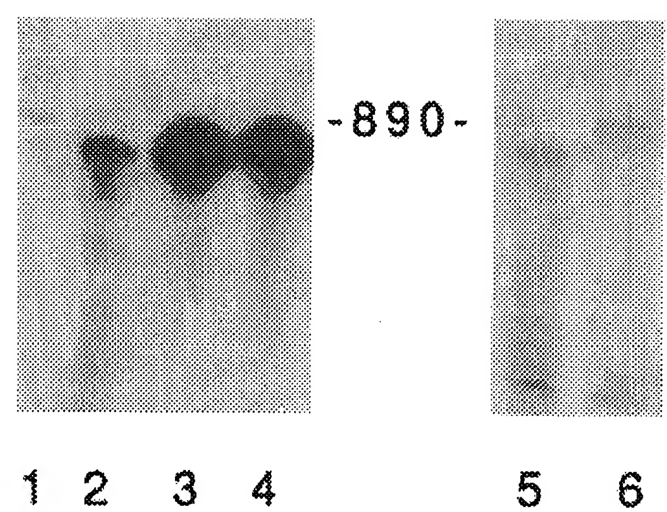


FIG. 11A

FIG. 11B

FIG. 12



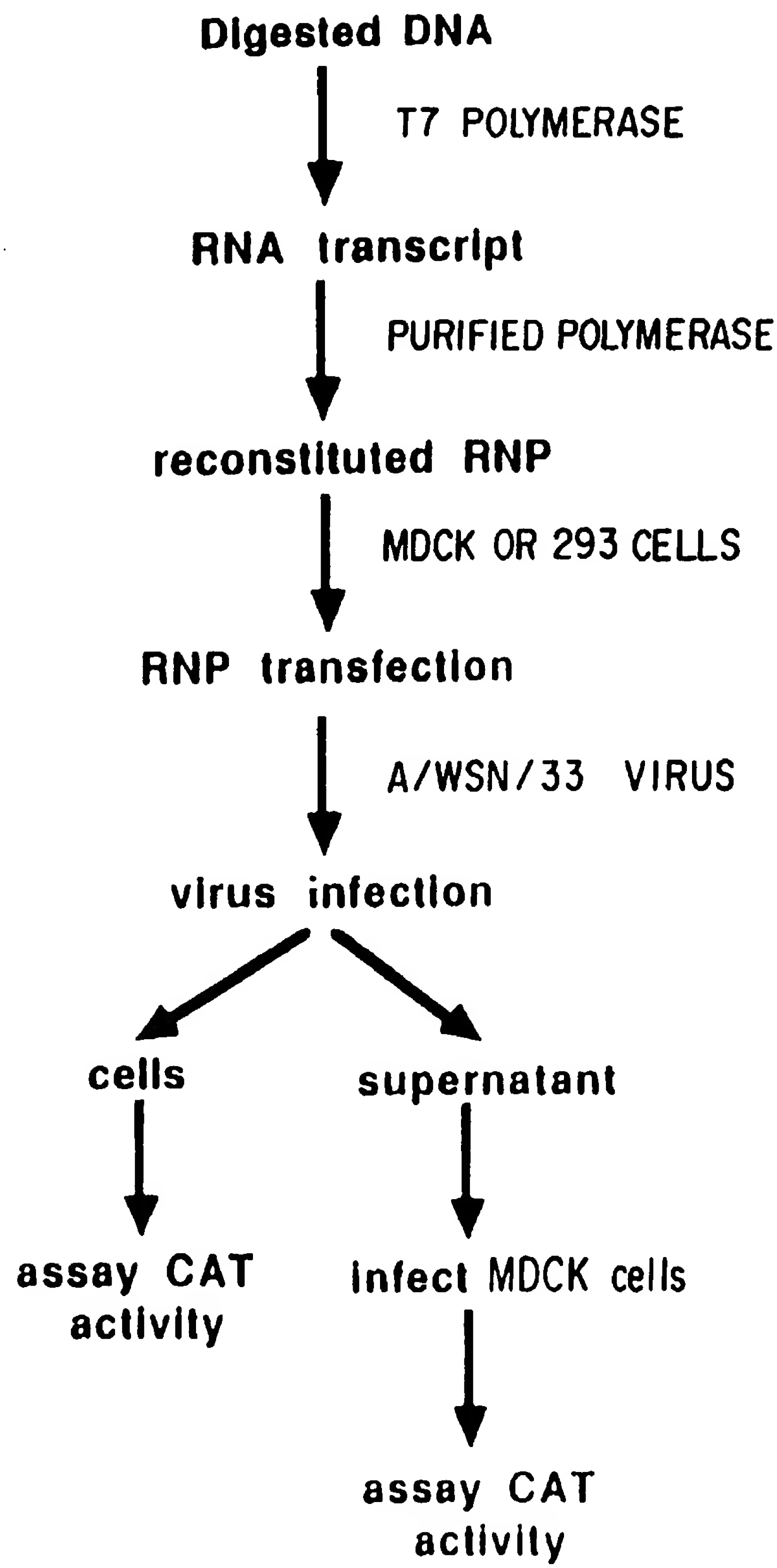

Digested DNA
T7 POLYMERASE
RNA transcript
PURIFIED POLYMERASE
reconstituted RNP
MDCK OR 293 CELLS
RNP transfection
A/WSN/33 VIRUS
virus infection
cells
supernatant
assay CAT activity
infect MDCK cells
assay CAT activity

FIG. 13

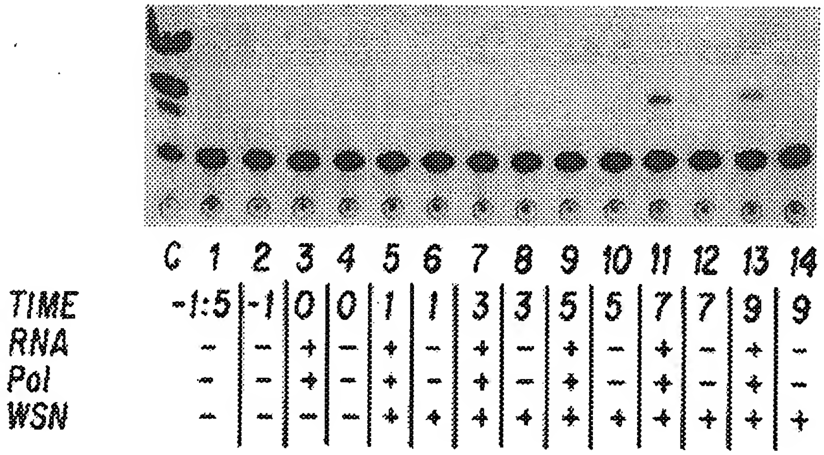


FIG. 14A

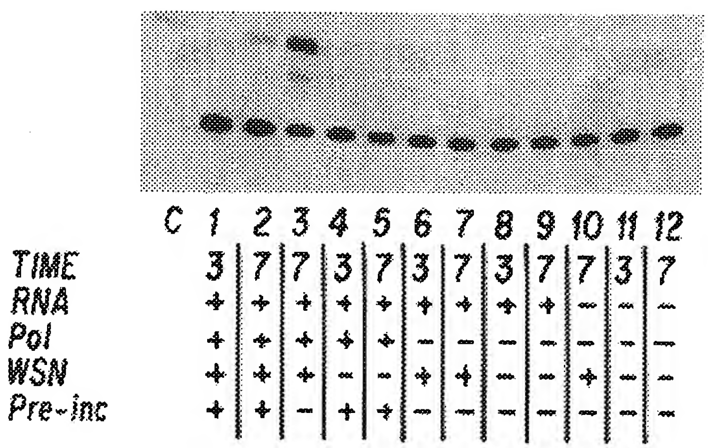


FIG. 14B

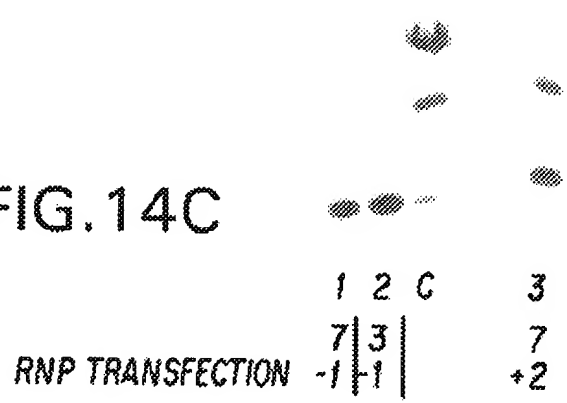
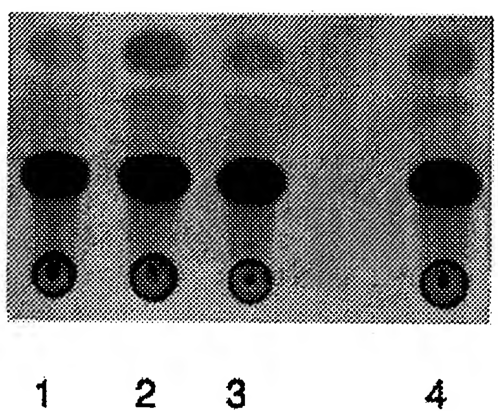


FIG. 14C

FIG. 15



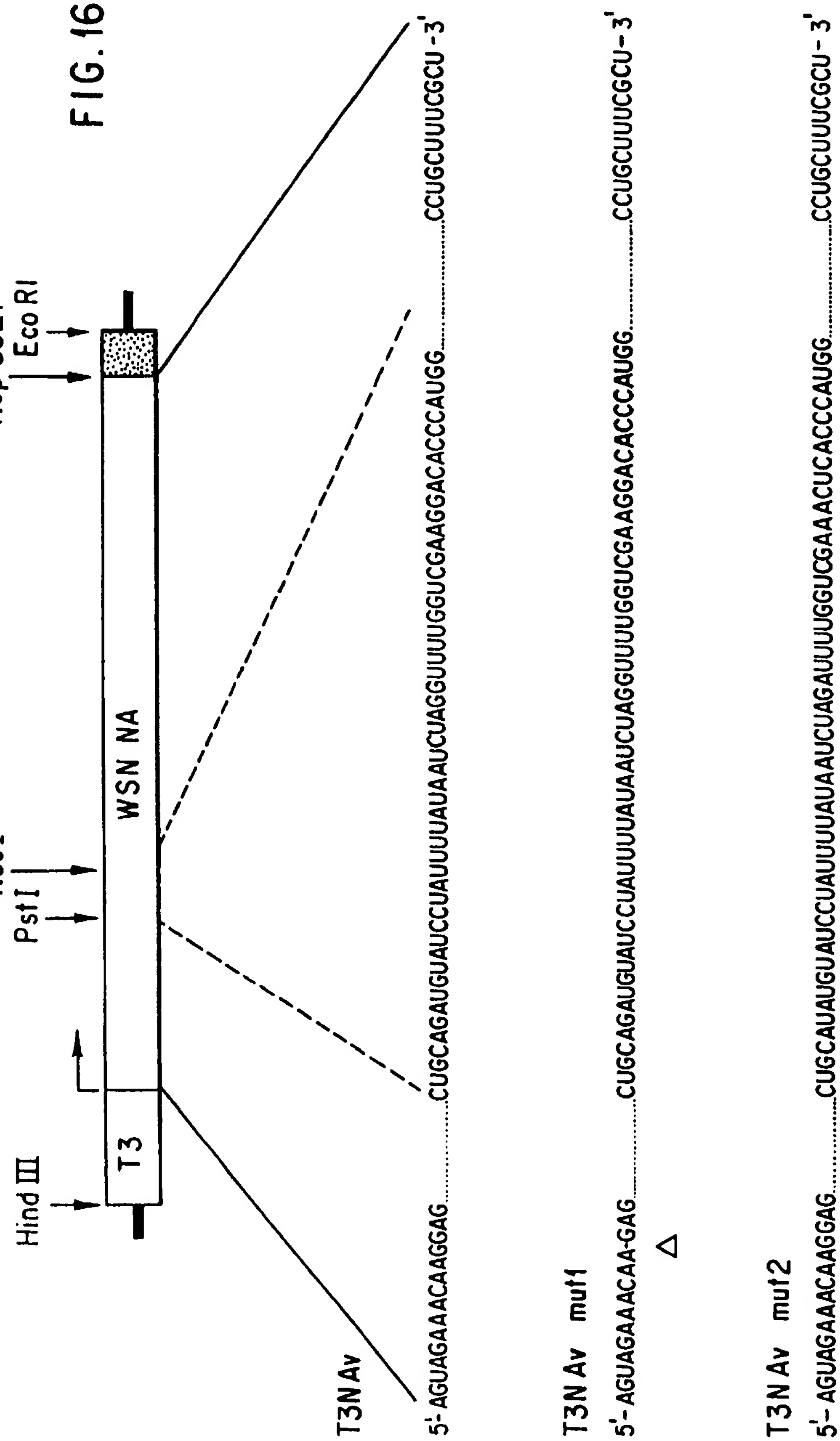

Ksp 632I
EcoRI
NcoI
PstI
HindIII
T3
WSN NA
T3NAv
5'-AGUAGAAACAAGGAG........CUGCAGAUGUAUCCUAUUUUUAUAAUCUAGGUUUUUGGUCGAAGGACACCCAUGG........CCUGCUUUUCGCU-3'
T3NAv mut1
5'-AGUAGAAACAA-GAG........CUGCAGAUGUAUCCUAUUUUUAUAAUCUAGGUUUUUGGUCGAAGGACACCCAUGG........CCUGCUUUUCGCU-3'
Δ
T3NAv mut2
5'-AGUAGAAACAAGGAG........CUGCAUAUGUAUCCUAUUUUUAUAAUCUAGAUUUUGGUCGAAACUCACCCAUGG........CCUGCUUUUCGCU-3'

FIG. 16

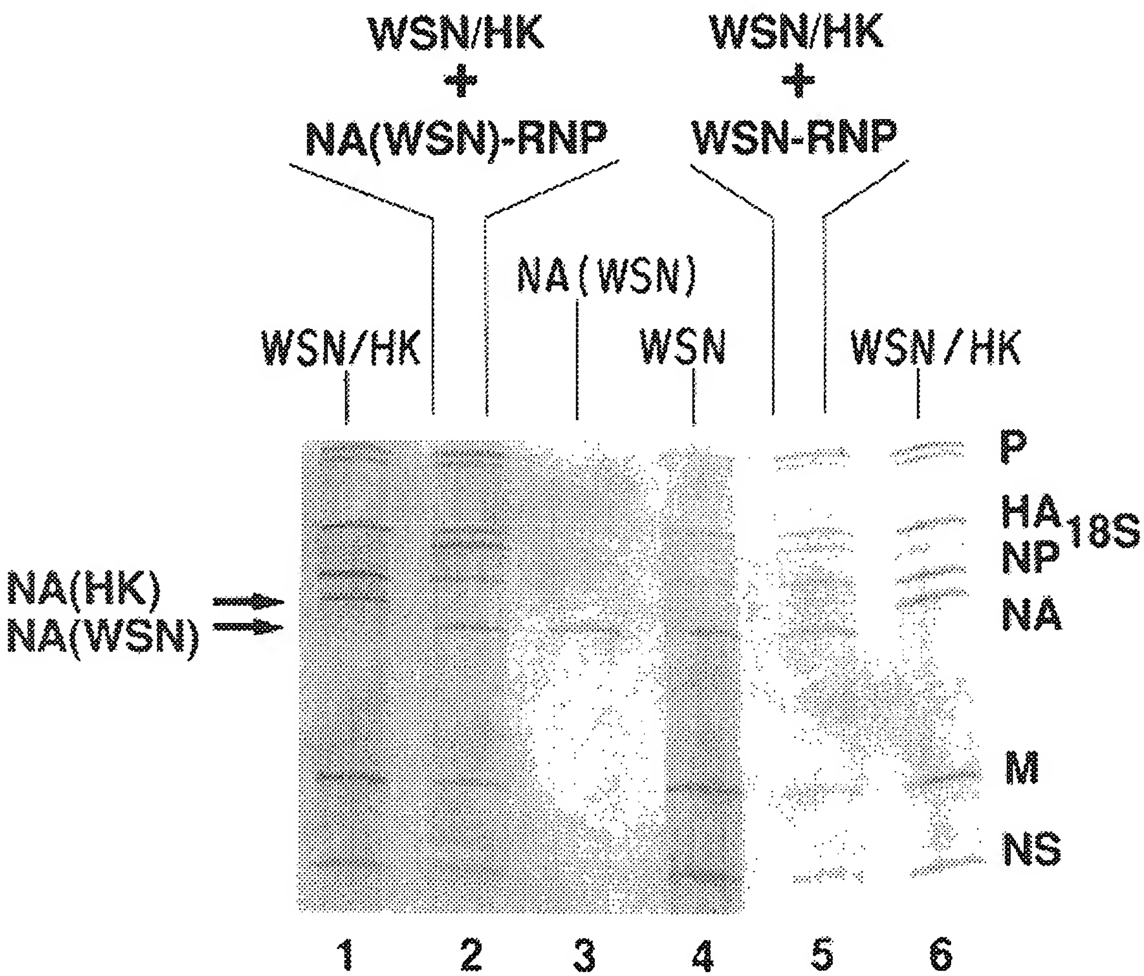

WSN/HK
+
NA(WSN)-RNP
WSN/HK
+
WSN-RNP
NA(WSN)
WSN/HK
WSN
WSN/HK
NA(HK)
NA(WSN)
P
HA
18S
NP
NA
M
NS
1
2
3
4
5
6

FIG. 17

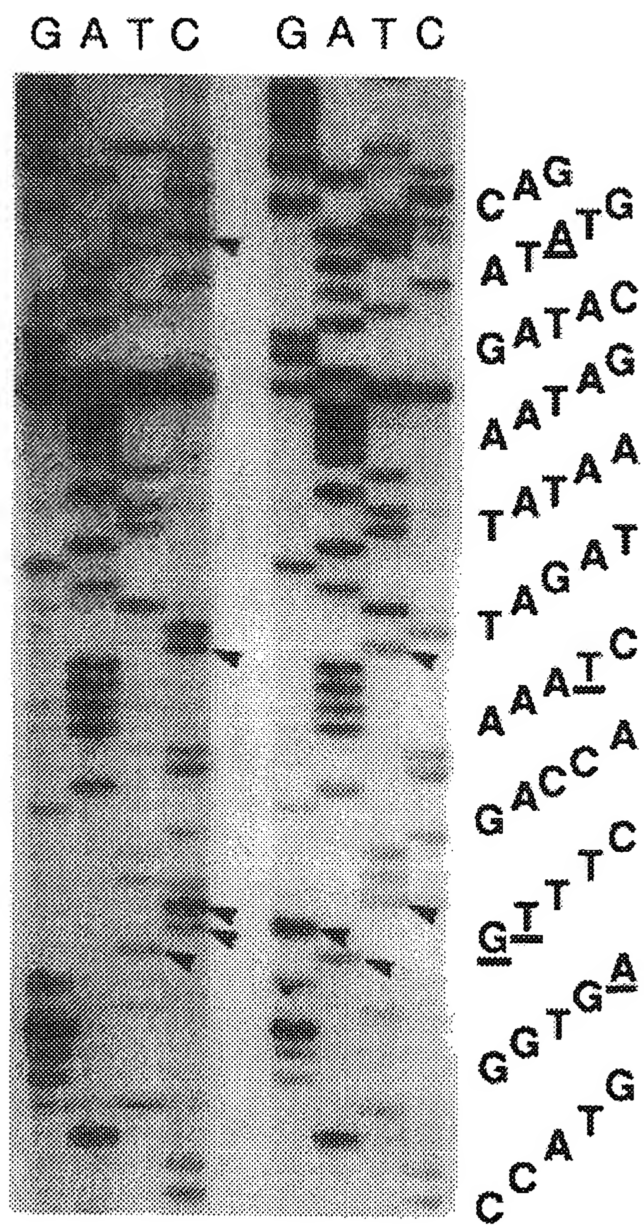
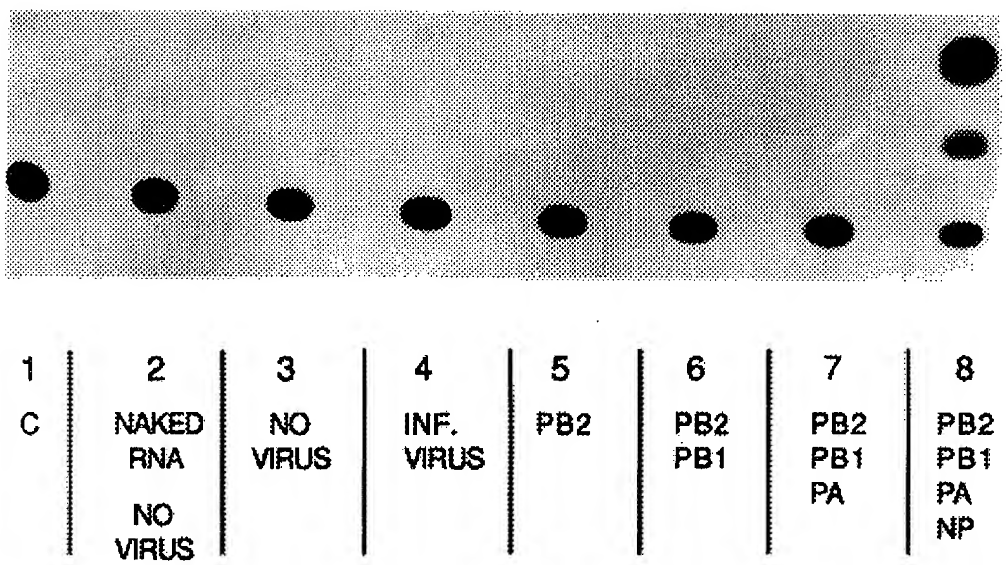

G A T C G A T C
CAG
ATATG
GATAC
AATAG
TATAA
TAGAT
AAATC
GACCA
GTTTC
GGTGA
CCATG

FIG. 18

FIG. 19


1
C
2
NAKED RNA
NO VIRUS
3
NO VIRUS
4
INF. VIRUS
5
PB2
6
PB2
PB1
7
PB2
PB1
PA
8
PB2
PB1
PA
NP

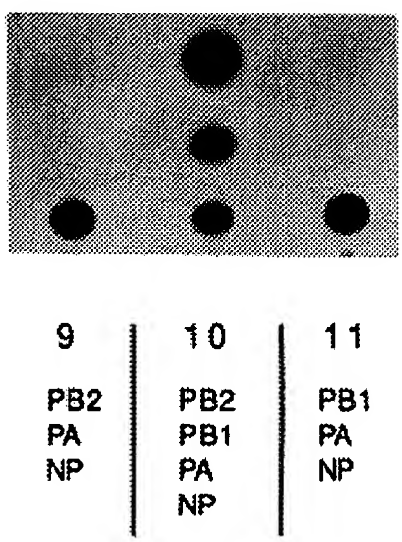

9
PB2
PA
NP
10
PB2
PB1
PA
NP
11
PB1
PA
NP

FIG.20A

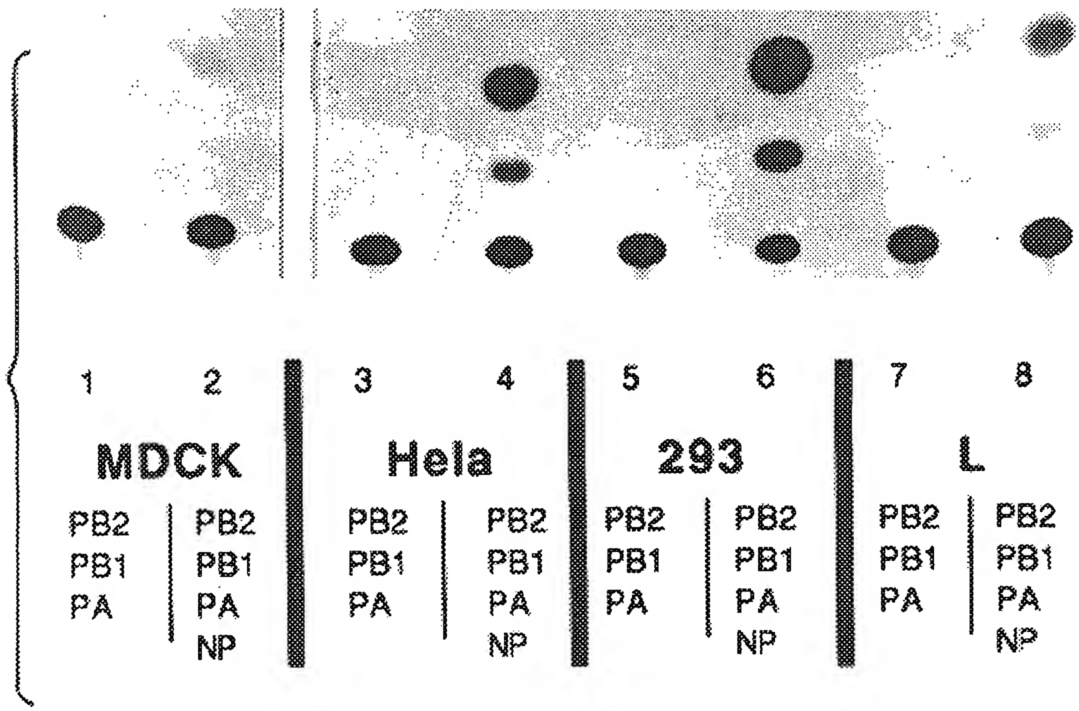
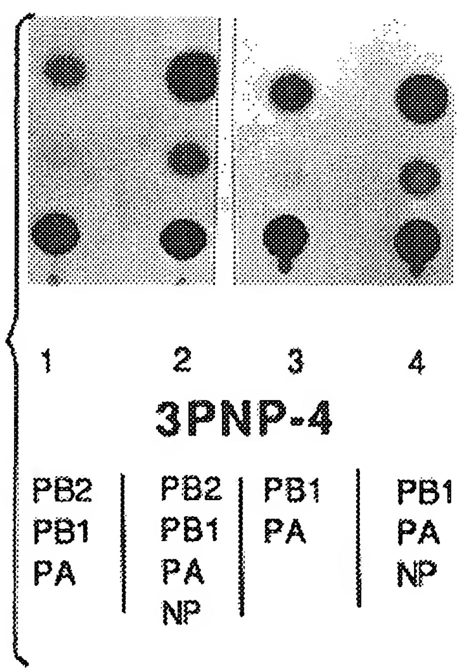


FIG.20B



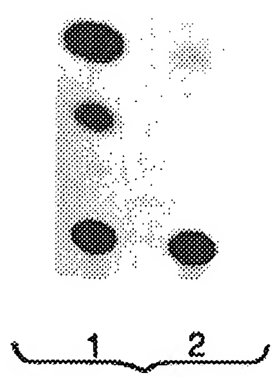
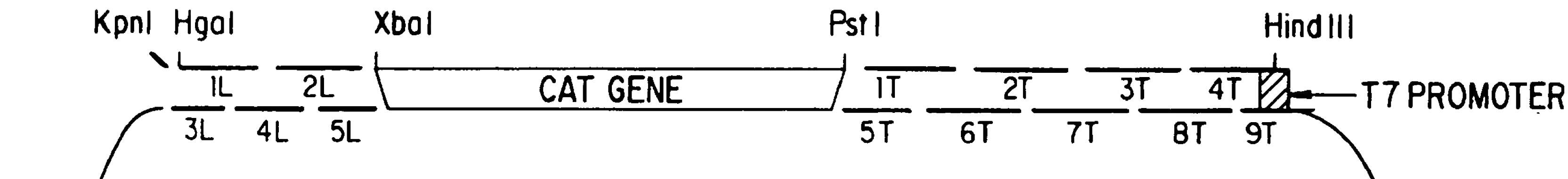


FIG.20C



Oligo Sequences:

Leader Oligos:

1L: 5' CGACGCATATTACGCGAAAAAATGCGTACAACAAACTTGCATAAAC
2L: 5' CAAAAAAATGGGGCAAATAAGAATTTGATAAGTACCACTTAAATTTAACT
3L: 5' CTAGAGTTAAATTTAAGTGGTACT
4L: 5' TATCAAATTCTTATTTGCCCCATTTTTTTGGTTTATGCAAGTTTGTTGTA
5L: 5' CGCATTTTTTCGCGTAATATGCGTCGGTAC

Trailer Oligos:

1T: 5' GTATTCAATTATAGTTATTAAAAATTAAAAATCATATAATTTTTTAAATA
2T: 5' ACTTTTAGTGAACTAATCCTAAAGTTATCATTTTAATCTTGGAGGAATAA
3T: 5' ATTTAAACCCTAATCTAATTGGTTTATATGTGTATTAACTAAATTACGAG
4T: 5' ATATTAGTTTTTGACACTTTTTTTCTCGTTATAGTGAGTCGTATTA
5T: 5' AGCTTAATACGACTCACTATAACGA
6T: 5' GAAAAAAAGTGTCAAAAACTAATATCTCGTAATTTAGTTAATACACATAT
7T: 5' AAACCAATTAGATTAGGGTTTAAATTTATTCCTCCAAGATTAAAATGATA
8T: 5' ACTTTAGGATTAGTTCACTAAAAGTTATTTAAAAAATTATATGATTTTTA
9T: 5' AATTTTAATAACTATAATTGAATACTGCA

FIG. 21

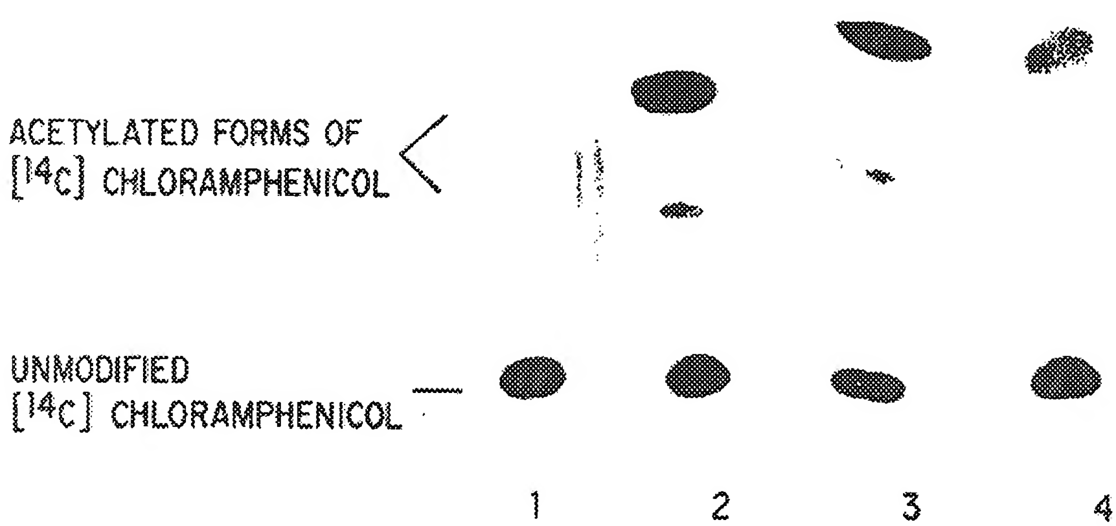
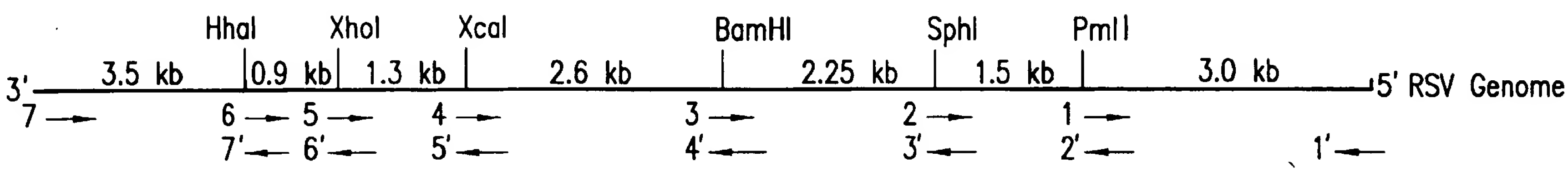

ACETYLATED FORMS OF
[14C] CHLORAMPHENICOL
UNMODIFIED
[14C] CHLORAMPHENICOL
1
2
3
4

FIG.22



Primer Sequences:

1: 5' GTTTAACACGTGGTGAG
2: 5' ACATATAGGCATGCACC
3: 5' GACAAAATGGATCCCATT
4: 5' TGGTTGGTATACCAGTGTT
5: 5' TACCAAGAGCTCGAGTCA
6: 5' TTTACCATATGCGCTAATGT
7: 5' ACGCGAAAAAATGCGTACA

1': 5' ACGAGAAAAAAAGTGTCAA
2': 5' CTCACCACGTGTTAAAC
3': 5' GGTGCATGCCTATATGT
4': 5' AATGGGATCCATTTTGTCC
5': 5' AACACTGGTATACCAACCA
6': 5' TGACTCGAGCTCTTGGTA
7': 5' ACATTAGCGCATATGGTAAA

FIG.23

RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS

This application is a continuation-in-part of application Ser. No. 08/190,698 filed Feb. 1, 1994, now abandoned, which is a continuation of Ser. No. 07/925,061 filed Aug. 4, 1992 (now abandoned) which is a divisional of Ser. No. 07/527,237 (now U.S. Pat. No. 5,166,057), which is a continuation-in-part of Ser. No. 07/440,053 filed Nov. 21, 1989 (now abandoned) and Ser. No. 07/399,728, filed Aug. 28, 1989, (now abandoned) which are incorporated by reference herein in their entirety.

1. INTRODUCTION

The present invention relates to recombinant negative strand virus RNA templates which may be used to express heterologous gene products in appropriate host cell systems and/or to construct recombinant viruses that express, package, and/or present the heterologous gene product. The expression products and chimeric viruses may advantageously be used in vaccine formulations.

The invention is demonstrated by way of examples in which recombinant influenza virus RNA templates containing a heterologous gene coding sequences in the negative-polarity were constructed. These recombinant templates, when combined with purified viral RNA-directed RNA polymerase, were infectious, replicated in appropriate host cells, and expressed the heterologous gene product at high levels. In addition, the heterologous gene was expressed and packaged by the resulting recombinant influenza viruses. The invention is further demonstrated by way of examples in which recombinant respiratory syncytial virus RNA templates containing a heterologous gene coding sequence in the negative-polarity were constructed and used to infect and replicate in appropriate host cells.

2. BACKGROUND OF THE INVENTION

A number of DNA viruses have been genetically engineered to direct the expression of heterologous proteins in host cell systems (e.g., vaccinia virus, baculovirus, etc.). Recently, similar advances have been made with positive-strand RNA viruses (e.g., poliovirus). The expression products of these constructs, i.e., the heterologous gene product or the chimeric virus which expresses the heterologous gene product, are thought to be potentially useful in vaccine formulations (either subunit or whole virus vaccines). One drawback to the use of viruses such as vaccinia for constructing recombinant or chimeric viruses for use in vaccines is the lack of variation in its major epitopes. This lack of variability in the viral strains places strict limitations on the repeated use of chimeric vaccinia, in that multiple vaccinations will generate host-resistance to the strain so that the inoculated virus cannot infect the host. Inoculation of a resistant individual with chimeric vaccina will, therefore, not induce immune stimulation.

By contrast, influenza virus, a negative-strand RNA virus, demonstrates a wide variability of its major epitopes. Indeed, thousands of variants of influenza have been identified; each strain evolving by antigenic drift. The negative-strand viruses such as influenza would be attractive candidates for constructing chimeric viruses for use in vaccines because its genetic variability allows for the construction of a vast repertoire of vaccine formulations which will stimulate immunity without risk of developing a tolerance. However, achieving this goal has been precluded by the fact that, to date, it has not been possible to construct recombinant or chimeric negative-strand RNA particles that are infectious.

2.1. The Influenza Virus

Virus families containing enveloped single-stranded RNA of the negative-sense genome are classified into groups having non-segmented genomes (Paramyxoviridae, Rhabdoviridae) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae). The Orthomyxoviridae family, described in detail below, and used in the examples herein, contains only the viruses of influenza, types A, B and C.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M). The segmented genome of influenza A consists of eight molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-directed RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix proteins (M1, M2); two surface glycoproteins which project from the lipoprotein envelope: hemagglutinin (HA) and neuraminidase (NA); and nonstructural proteins whose function is unknown (NS 1 and NS2). Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding on the plasma membrane. The viruses can reassort genes during mixed infections.

Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed as the essential initial event in infection. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'-terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act as signals for the template-independent addition of poly(A) tracts. Of the eight viral mRNA molecules so produced, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NS2. In other words, the eight viral mRNAs code for ten proteins: eight structural and two nonstructural. A summary of the genes of the influenza virus and their protein products is shown in Table I below.

TABLE I

Influenza Virus Genome RNA Segments and Coding Assignments[a]

Segment	Length[b] (Nucleotides)	Encoded Polypeptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
1	2341	PB2	759	30–60	RNA transcriptase component; host cell RNA cap binding
2	2341	PB1	757	30–60	RNA transcriptase component; initiation of transcription; endonuclease activity?

TABLE I-continued

Influenza Virus Genome RNA Segments and Coding Assignments[a]

Segment	Length[b] (Nucleotides)	Encoded Polypeptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
3	2233	PA	716	30–60	RNA transcriptase component; elongation of mRNA chains?
4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
5	1565	NP	498	100	Nucleoprotein; associated with RNA; structural component of RNA transcriptase
6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
7	1027	M_1	252	3000	Matrix protein; lines inside of envelope
		M_2	96		Structural protein in plasma membrane; spliced mRNA
		?	?9		Unidentffled protein
8	890	NS_1	230		Nonstructural protein; function unknown
		NS_2	121		Nonstructural protein; function unknown; spliced mRNA

[a]Adapted from R. A. Lamb and P. W. Choppin (1983), Reproduced from the Annual Review of Biochemistry, Volume 52, 467–506.
[b]For A/PR/8/34 strain.
[c]Determined by biochemical and genetic approaches.
[d]Determined by nucleotide sequence analysis and protein sequencing.

Following transcription, virus genome replication is the second essential event in infection by negative-strand RNA viruses. As with other negative-strand RNA viruses, virus genome replication in influenza is mediated by virus-specified proteins. It is hypothesized that most or all of the viral proteins that transcribe influenza virus mRNA segments also carry out their replication. All viral RNA segments have common 3' and 5' termini, presumably to enable the RNA-synthesizing apparatus to recognize each segment with equal efficiency. The mechanism that regulates the alternative uses (i.e., transcription or replication) of the same complement of proteins (PB2, PB1, PA and NP) has not been clearly identified but appears to involve the abundance of free forms of one or more of the nucleocapsid proteins, in particular, the NP. The nucleus appears to be the site of virus RNA replication, just as it is the site for transcription.

The first products of replicative RNA synthesis are complementary copies (i.e., plus-polarity) of all influenza virus genome RNA segments (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand mRNA transcripts in the structure of their termini. Unlike the MRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5' termini, and are not truncated and polyadenylated at the 3' termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in each genomic RNA segment in the complementary form. The cRNAs serve as templates for the synthesis of genomic negative-strand vRNAs.

The influenza virus negative strand genomes (vRNAs) and antigenomes (cRNAs) are always encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. In contrast to the other enveloped RNA viruses, nucleocapsid assembly appears to take place in the nucleus rather than in the cytoplasm. The virus matures by budding from the apical surface of the cell incorporating the M protein on the cytoplasmic side or inner surface of the budding envelope. The HA and NA become glycosylated and incorporated into the lipid envelope. In permissive cells, HA is eventually cleaved, but the two resulting chains remain united by disulfide bonds.

It is not known by what mechanism one copy of each of the eight genomic viral RNAs is selected for incorporation into each new virion. Defective interfering (DI) particles are often produced, especially following infection at high multiplicity.

2.2. RNA Directed RNA Polymerase

The RNA-directed RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase could only be studied by inference using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid- derived RNA is introduced into cells by transfection (for example, Racaniello et al. 1981, Science 214: 916–919; Levis, et al., 1986, Cell 44: 137–145). In the case of poliovirus, the purified polymerase will replicate a genome RNA in in vitro reactions and when this preparation is transfected into cells it is infectious (Kaplan, et al., 1985, Proc. Natl. Acad. Sci. USA 82: 8424–8428). However, the template elements which serve as transcription promoter for the poliovirus-encoded polymerase are unknown since even RNA homopolymers can be copied (Ward, et al., 1988, J. Virol. 62: 558–562). SP6 transcripts have also been used to produce model defective interfering (DI) RNAs for the Sindbis viral genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis viral polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5' terminus and 19 nt of the 3' terminus of the genome (Levis, et al., 1986, Cell 44: 137–145). In the case of brome mosaic virus (BMV), a positive strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134 nt tRNA-like 3' terminus (Dreher, and Hall, 1988, J. Mol. Biol. 201: 31–40). Polymerase recognition and synthesis were shown to be dependent on both sequence and secondary structural features (Dreher, et al., 1984, Nature 311: 171–175).

The negative-sense RNA viruses have been refractory to study of the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is only active in transcription when virus-derived ribonucleoprotein complexes (RNPs) are included as template (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 126: 40–49); Emerson and Yu, 1975, J. Virol. 15: 1348–1356; Naito, and Ishihama, 1976, J. Biol. Chem. 251: 4307–4314). RNPs have been reconstituted from naked RNA of VSV DI particles using infected cell extracts as protein source. These RNPs were then replicated when added back to infected cells (Mirakhur, and Peluso, 1988, Proc. Natl. Acad. Sci. USA 85: 7511–7515). With regard to influenza viruses, it

was recently reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid and polymerase proteins were gel-purified and renatured on the viral RNA using thioredoxin (Szewczyk, et al., 1988, Proc. Natl. Acad. Sci. USA, 85: 7907–7911). However, these authors did not show that the activity of the preparation was specific for influenza viral RNA, nor did they analyze the signals which promote transcription.

During the course of influenza virus infection the polymerase catalyzes three distinct transcription activities. These include the synthesis of (a) subgenomic mRNA, which contains a 5' cap and a 3' poly-A tail; (b) a full length plus-strand or anti-genome (cRNA) copied from the genome RNA; and (c) genomic vRNA synthesized from the full length cRNA (reviewed in Ishihama and Nagata, 1988, CRC Crit. Rev. Biochem. 23: 27–76; and Krug, Transcription and replication of influenza viruses. In: Genetics of influenza viruses, Ed., Palese, P. and Kingsbury, D. W. New York, Springer-Verlag, 1983, p. 70–98). Viral proteins PB2, PB1 and PA are thought to catalyze all influenza virus-specific RNA synthesis when in the presence of excess nucleocapsid protein (NP; see above reviews). These polymerase functions have been studied using RNP cores derived from detergent-disrupted virus, and RNPs from the nuclear extracts of infected cells. Transcription from the RNPs derived from disrupted virus occurs when primed with either dinucleotide adenylyl-(3'–5')-guanosine (ApG) or capped mRNAs. The plus sense mRNA products have terminated synthesis 17–20 nucleotides upstream of the 5' terminus of the RNA template and have been processed by the addition of poly A tails. These products cannot serve as template for the viral-sense genome since they lack terminal sequences (Hay, et al., 1977, Virology 83: 337–355). RNPs derived from nuclear extracts of infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers. However, if ApG is used under these conditions, both RNAs, polyadenylated and full length cRNA, can be obtained (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282–6286; Takeuchi, et al., 1987, J. Biochem. 101: 837–845). Recently it was shown that replicative synthesis of cRNA could occur in the absence of exogenous primer if the nuclear extract was harvested at certain times post infection. In these same preparations the synthesis of negative-sense vRNA from a cRNA template was also observed (Shapiro and Krug, 1988, J. Virol. 62: 2285–2290). The synthesis of full length cRNA was shown to be dependent upon the presence of nucleocapsid protein (NP) which was free in solution (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282–6286; Shapiro and Krug, 1988, J. Virol. 62: 2285–2290). These findings led to the suggestion that the regulatory control between mRNA and cRNA synthesis by the RNP complex is based on the requirement for there being an excess of soluble NP (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282–6286).

Another line of investigation has focused on the preparation of polymerase-RNA complexes derived from RNPs from detergent-disrupted virus. When the RNP complex is centrifuged through a CsCl-glycerol gradient, the RNA can be found associated with the three polymerase (P) proteins at the bottom of the gradient. Near the top of the gradient, free NP protein can be found (Honda, et al., 1988, J. Biochem. 104: 1021–1026; Kato, et al., 1985, Virus Research 3, 115–127). The purified polymerase-RNA complex (bottom of gradient), is active in initiating ApG-primed synthesis of RNA, but fails to elongate to more than 12–19 nucleotides. When fractions from the top of the gradient containing the NP protein are added back to the polymerase-RNA complex, elongation can ensue (Honda, et al., 1987, J. Biochem. 102: 41–49). These data suggest that the NP protein is needed for elongation, but that initiation can occur in the absence of NP.

It has been shown that the genomic RNA of influenza viruses is in a circular conformation via base-pairing of the termini to form a panhandle of 15 to 16 nt (Honda, et al., 1988, J. Biochem. 104: 1021–1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140–8144). Since the viral polymerase was found bound to the panhandle, this led to the suggestion that a panhandle structure was required for recognition by the viral polymerase (Honda, et al., 1988, J. Biochem. 104: 1021–1026). Therefore, it was hypothesized in these two reports that the promoter for the viral RNA polymerase was the double stranded RNA in panhandle conformation.

3. SUMMARY OF THE INVENTION

Recombinant negative-strand viral RNA templates are described which may be used with purified RNA-directed RNA polymerase complex to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. Both influenza and respiratory syncytial viral templates are exemplified. The RNA templates are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template.

As demonstrated by the examples described herein, recombinant negative-sense influenza RNA templates may be mixed with purified viral polymerase proteins and nucleoprotein (i.e., the purified viral polymerase complex) to form infectious recombinant RNPs. These can be used to express heterologous gene products in host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with recombinant RNPs and virus. Alternatively, the recombinant RNA templates or recombinant RNPs may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. Additionally, a non-virus dependent replication system for influenza virus is also described. Vaccinia vectors expressing influenza virus polypeptides were used as the source of proteins which were able to replicate and transcribe synthetically derived RNPs. The minimum subset of influenza virus protein needed for specific replication and expression of the viral RNP was found to be the three polymerase proteins (PB2, PB1 and PA) and the nucleoprotein (NP). This suggests that the nonstructural proteins, NS1 and NS2, are not absolutely required for the replication and express of viral RNP.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The use of recombinant influenza for this purpose is especially attractive since influenza demonstrates tremendous strain variability allowing for the construction of a vast repertoire of vaccine formulations. The ability to select from thousands of influenza variants for constructing chimeric viruses obviates the problem of host resistance encountered when using other viruses such as vaccinia. In addition, since influenza stimulates a vigorous secretory and cytotoxic T cell response, the presentation of foreign epitopes in the influenza virus background may also provide for the induction of secretory immunity and cell-mediated immunity.

3.1. Definitions

As used herein, the following terms will have the meanings indicated:

cRNA=anti-genomic RNA

HA=hemagglutinin (envelope glycoprotein)

M=matrix protein (lines inside of envelope)

MDCK=Madin Darby canine kidney cells

MDBK=Madin Darby bovine kidney cells

moi=multiplicity of infection

NA=neuraminidase (envelope glycoprotein)

NP=nucleoprotein (associated with RNA and required for polymerase activity)

NS=nonstructural protein (function unknown)

nt=nucleotide

PA, PB1, PB2 =RNA-directed RNA polymerase components

RNP=ribonucleoprotein (RNA, PB2, PB1, PA and NP)

rRNP=recombinant RNP

vRNA=genomic virus RNA

viral polymerase complex=PA, PB1, PB2 and NP

WSN=influenza A/WSN/33 virus

WSN-HK virus: reassortment virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus

4. DESCRIPTION OF THE FIGURES

FIG. 1. Purification of the polymerase preparation. RNP cores were purified from whole virus and then subjected to CsCl-glycerol gradient centrifugation. The polymerase was purified from fractions with 1.5 to 2.0M CsCl. Samples were then analyzed by polyacrylamide gel electrophoresis on a 7–14% linear gradient gel in the presence of 0.1% sodium dodecylsulfate followed by staining with silver. Protein samples contained 1.4 μg whole virus (lane 1), 0.3 μg whole virus (lane 2), 5 μl of RNP cores (lane 3) and 25 μl RNA polymerase (lane 4). Known assignments of the proteins are indicated at the left.

FIG. 2. Plasmid constructs used to prepare RNA templates. The plasmid design is depicted with the solid box representing pUC-19 sequences, the hatched box represents the truncated promoter specifically recognized by bacteriophage T7 RNA polymerase, the solid line represents the DNA which is transcribed from plasmids which have been digested with MboII. The white box represents sequences encoding the recognition sites for MboII, EcoRI and PstI, in that order. Sites of cleavage by restriction endonucleases are indicated. Beneath the diagram, the entire sequences of RNAs which result from synthesis by T7 RNA polymerase from MboII-digested plasmid are given. The V-wt RNA has the identical 5' and 3' termini as found in RNA segment 8 of influenza A viruses, separated by 16 “spacer” nucleotides. The RNA, M-wt, represents the exact opposite stand, or “message-sense”, of V-wt. Restriction endonuclease sites for DraI EcoRI, PstI and SmaI are indicated. T7 transcripts of plasmids cleaved by these enzymes result in, respectively, 32, 58, 66 and 91 nucleotide long RNAs. The sequences of V-d5' RNA are indicated. The plasmid design is essentially the same as that used for the V-wt RNA except for the minor changes in the “spacer” sequence. The point mutants of V-d5' RNAs which were studied are indicated in Table I.

FIG. 3. Analysis of products of influenza viral polymerase. FIG. 3A: Polymerase reaction mixtures containing 0.4 mM ApG (lane 2) or no primer (lane 3) were electrophoresed on 8% polyacrylamide gels containing 7.7M urea. FIG. 3B: The nascent RNA is resistant to single-stranded specific nuclease S1. Following the standard polymerase reaction, the solutions were diluted in nuclease S1 buffer (lane 1) and enzyme was added (lane 2). As control for S1 digestion conditions, radioactively labeled single-stranded V-wt RNA was treated with nuclease S1 (lane 3) or with buffer alone (lane 4). FIG. 3C: Ribonuclease T1 analysis of gel-purified reaction products. The reaction products of the viral polymerase using the V-wt RNA template was subjected to electrophoresis on an 8% polyacrylamide gel. The 53 nt band and the smaller transcript were excised and eluted from the gel matrix. These RNAs were digested with RNAse T1 and analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.7M urea. For comparison, T7 transcripts of M-wt and V-wt RNAs which had been synthesized in the presence of α-^{32}P-UTP were also analyzed with RNAse T1. The predicted radiolabeled oliognucleotides of the control RNAs are indicated. Lane 1, 53 nucleotide full length (FL) product; lane 2, 40–45 nucleotide smaller (Sm) RNA product; lane 3, M-wt RNA labeled by incorporation of ^{32}P-MP; and lane 4, V-wt RNA labeled as in lane 3.

FIG. 4. Optimal reaction conditions for the viral polymerase. FIG. 4A: Reactions with V-wt template were assembled on ice and then incubated at the indicated temperatures for 90 minutes. FIG. 4B: Reactions with the V-wt template were prepared in parallel with the indicated NaCl or KCl concentrations and were incubated at 30° C. for 90 minutes. FIG. 4C: A single reaction with the V-wt template was incubated at 30° C., and at the indicated times, samples were removed and immediately processed by phenol-chloroform extraction. All gels contained 8% polyacrylamide with 7.7M urea.

FIG. 5. Template specificity of the viral polymerase. FIG. 5A: The viral polymerase reaction requires 3' terminal promoter sequences. Different template RNAs were used in reactions under standard conditions. Lane 1, the V-Pst RNA, which is identical to V-wt except it has a 13 nt extension at the 3' end; lane 2, V-Sma RNA, which has a 38 nt extension at the 3' end; lane 3, V-wt RNA; lane 4, a DNA polynucleotide with identical sequence as the V-wt RNA; lane 5, and 80 nt RNA generated by bacteriophage T3 RNA polymerase transcription of a pIBI-31 plasmid digested with HindIII. The autoradiograph was overexposed in order to emphasize the absence of specific reaction products when these other templates were used. FIG. 5B: 10 ng of each template RNA were incubated with the viral polymerase and the products were then subjected to electrophoresis on 8% polyacrylamide gels containing 7.7M urea. Lane 1, V-wt RNA; lane 2, V-Dra RNA; lane 3, V-Eco RNA; lane 4, M-wt RNA are shown; and lane 5, a 53nt marker oligonucleotide.

For the exact sequence differences refer to FIG. 2 and Section 6.1 et seq.

FIG. 6. The RNA promoter does not require a terminal panhandle. Polymerase reaction using two template RNAs. Each reaction contained 5 ng of V-wt RNA. As a second template the reactions contained 0 ng (lane 1), 0.6 ng (lane 2), and 3.0 ng (lane 3) of V-d5' RNA. The resulting molar ratios are as indicated in the figure. The reaction products were analyzed on an 8% polyacrylamide gel in the presence of 7.7M urea. Following densitometry analysis of autoradiographs, the relative intensity of each peak was corrected for the amount of radioactive UMP which is incorporated in each product.

FIG. 7. Specificity of promoter sequences. RNAs which lacked the 5' terminus and contained point mutations (Table

II) were compared with V-d5' RNA in standard polymerase reactions. The right panel is from a separate reaction set. Quantitative comparisons is outlined in Table II.

FIG. **8**. High concentration polymerase preparations are active in cap-endonuclease primed and in primeness RNA synthesis reactions. FIG. **8**A: Primer specificity of the high concentration enzyme. Radioactively synthesized 30 nt template is in lane 1. Reactions using 20 ng of V-d5' RNA and 5 μl of viral polymerase contained as primer: no primer (lane 2); 100 ng BMV RNA (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 6: 40–49) containing a cap 0 structure (lane 3); 100 ng rabbit globin mRNA, containing a cap 1 structure, (lane 4); and 0.4 mM ApG (lane 5). A lighter exposure of lane 5 is shown as lane 6. FIG. **8**B: Nuclease S1 analysis of gel-purified RNAs. Products from reactions using as primer ApG (lanes 1 and 2); no primer (lanes 3 and 4); or globin mRNA (lanes 5 and 6) were electrophoresed in the absence of urea and the appropriate gel piece was excised and the RNA was eluted. This RNA was then digested with nuclease S1 (lanes 2, 4, and 6) and the products were denatured and analyzed on an 8% polyacrylamide gel containing 7.7M urea.

FIG. **9**. Genomic length RNA synthesis from reconstituted RNPs. Reaction products using 10 μl of polymerase and as template 890 nt RNA identical to the sequence of segment 8 of virus A/WSN/33 and RNA extracted from A/PR/8/34 virus were analyzed on a 4% polyacrylamide gel containing 7.7M urea. In lane 1, the 890 nt template synthesized radioactively by T7 RNA polymerase is shown. The 890 nt plasmid-derived RNA was used as template in lanes 2, 3, 8 and 9. RNA extracted from virus was used as template in lanes 4, 5, 10 and 11. No template was used in lanes 6 and 7. No primer was used in lanes 2 to 5, and ApG was used as primer in lanes 6 to 11. Reaction products were treated with nuclease S1 in lanes 3, 5, 7, 9 and 11.

FIG. **10**. Diagrammatic representation of a PCR-directed mutagenesis method which can be used to replace viral coding sequences within viral gene segments.

FIG. **11**. (A). Diagrammatic representation of relevant portions of pIVCAT1. The various domains are labeled and are, from left to right; a truncated T7 promoter; the 5' nontranslated end of influenza A/PR/8/34 virus segment 8 (22 nucleotides); 8 nucleotides of linker sequence; the entire CAT gene coding region (660 nucleotides) the entire 3' nontranslated end of influenza A/PR/8/34 virus segment 8 (26 nucleotides); and linker sequence containing the HgaI restriction enzyme site. Relevant restriction enzyme sites and start and stop sites for the CAT gene are indicated. (B) The 716 base RNA product obtained following HgaI digestion and transcription of pIVACAT1 by T7 RNA polymerase. Influenza viral sequences are indicated by bold letters, CAT gene sequences by plain letters, and linker sequences by italics. The triplets—in antisense orientation—representing the initiation and termination codons of the CAT gene are indicated by arrow and underline, respectively.

FIG. **12**. RNA products of T7 polymerase transcription and in vitro influenza virus polymerase transcription. Lanes 1–4: polyacrylamide gel analysis of radiolabeled T7 polymerase transcripts from pIVACAT1, and pHgaNS. Lanes 5 and 6: Polyacrylamide gel analysis of the radiolabeled products of in vitro transcription by purified influenza A polymerase protein using unlabeled 1VACAT1 RNA and HgaNS RNA templates. Lane 1: HgaNS RNA of 80 nt. Lanes 2–4: different preparations of IVACAT1 RNA. Lane 5: viral polymerase transcript of IVACAT1 RNA. Lane 6: viral polymerase transcript of HgaNS RNA.

FIG. **13**. Schematic of the RNP-transfection and passaging experiments.

FIG. **14**. CAT assays of cells RNP-transfected with IVACAT1 RNA. (A) Time course of RNP-transfection in 293 cells. Cells were transfected at −1 hour with the recombinant RNP and infected with virus at 0 hour. Cells were harvested at the indicated time points and assayed for CAT activity. (B) Requirements for RNP-transfection of 293 cells Parameters of the reaction mixtures were as indicated. (C) RNP-transfection of MDCK cells. MDCK cells were transfected with IVACAT1 RNA-polymerase at either −1 hour or +2 hours relative to virus infection. Cells were harvested and CAT activity assayed at the indicated times. Components/conditions of the reaction were as indicated. "Time" indicates the time point of harvesting the cells. T=0 marks the time of addition of helper virus. "RNA" represents the IVACAT1 RNA. "Pol" is the purified influenza A/PR/8/34 polymerase protein complex. "WSN" indicates the influenza A/WSN/33 helper virus. "Pre-Inc." indicates preincubation of RNA and polymerase in transcription buffer at 30° C. for 30 min. "RNP transfection" indicates the time of RNP transfection relative to virus infection. "+/−" indicate presence or absence of the particular component/feature. "C" indicates control assays using commercially available CAT enzyme (Boehringer-Mannheim).

FIG. **15**. CAT activity in MDCK cells infected with recombinant virus. Supernatant from RNP-transfected and helper virus-infected MDCK cells was used to infect fresh MDCK cells. The inoculum was removed 1 hour after infection, cells were harvested 11 hours later and CAT activity was assayed. Lane 1: extract of cells infected with helper virus only. Lane 2: extract of cells infected with 100 μl of supernatant from RNP-transfected and helper virus-infected MDCK cells. Lane 3: Supernatant (80 μl) of cells from lane 2. Lane 4: Same as lane 2 except that helper virus (MOI 4) was added to inoculum. In contrast to experiments shown in FIG. **4**, the assays contained 20 μl of ^{14}C chloramphenicol.

FIG. **16**. Diagram of relevant portions of the neuraminidase (NA) gene contained in plasmids used for transfection experiments. The pUC19 derived plasmid pT3NAv contains the influenza A/WSN/33 virus NA gene and a truncated promoter specifically recognized by bacteriophage T3 RNA polymerase. The T3 promoter used is truncated such that the initial transcribed nucleotide (an adenine) corresponds to the 5' adenine of the WSN NA gene. At the 3' end of the cDNA copy of the NA gene, a Ksp632I restriction enzyme site was inserted such that the cleavage site occurs directly after the 3' end of the NA gene sequence. A 1409 nucleotide long transcript was obtained following Ksp632I digestion and transcription by T3 RNA polymerase of PT3NAv (as described in Section 8.1, infra). The 15 5' terminal nucleotides, the 52 nucleotides corresponding to the region between the restriction endonuclease sites NcoI and PstI and the 12 3' terminal nucleotides are shown. The transcript of pT3NAv mut 1 is identical to that of pT3NAv except for a single deletion, eleven nucleotides downstream from the 5' end of the wild type RNA. The transcript of the pT3NAv mut 2 is identical to that of pT3NAv except for 5 mutations located in the central region (indicated by underline). These five mutations do not change the amino acid sequence in the open reading frame of the gene. The serine codon UCC at position 887–889 (plus sense RNA) was replaced with the serine codon AGU in the same frame. The numbering of nucleotides follows Hiti et al., 1982, J. Virol., 41: 730–734.

FIG. **17**. Polyacrylamide gel electrophoresis of RNAs purified from rescued influenza viruses. RNA transcripts of

pT3NAs (FIG. 16) of phenol-extracted RNA derived from influenza A/WSN/33 virus was mixed with purified polymerase preparations following the protocol described in Section 6.1.1, infra. These reconstituted RNPs were then transfected into MDBK cells which had been infected one hour earlier with WSN-HK helper virus. The medium, containing 28 μg/ml plasminogen, was harvested after 16 hours and virus was amplified and plaqued on MDBK cells in the absence of protease. Virus obtained from plaques was then further amplified in MDBK cells and RNA was phenol-extracted from purified virus preparations as described in Sections 6.1 et seq. and 7.1 et seq. RNAs were separated on 2.8% polyacrylamide-0.075 % bisacrylamide gels containing 7.7M urea in TBE buffer and visualized by silverstaining as described in Section 6.1 et seq. Lanes 1 and 6: WSN-HK virus RNA. Lane 2: RNA of virus which was rescued from MDBK cells following RNP-transfection with pT3NAv derived NA RNA and infection with helper virus WSN-HK. Lane 3: NA RNA transcribed in vitro from pT3NAv. Lane 4: RNA of control WSN virus. Lane 5: RNA of virus which was rescued from MDBK cells following RNP-transfection with phenol-extracted WSN virus RNA and infection with helper virus WSN-HK.

FIG. 18. Sequence analysis of RNA obtained from rescued influenza virus containing five site-specific mutations. Following infection with the WSN-HK helper virus, MDBK cells were RNP-transfected with T3NAv mut 2 RNA which was obtained by transcription from pT3NAv mut 2. Following overnight incubation in the presence of 28 μg/ml plasminogen, medium was used for propagation and plaquing on MDBK cells in the absence of protease. Virus from plaques was then amplified and RNA was obtained following phenol-extraction of purified virus. Rescue of the mutant NA gene into virus particles was verified through direct RNA sequencing using 5'-TACGAGGAAATGTTCCTGTTA-3' as primer (corresponding to position 800–819; Hiti et al., J. Virol., 41: 730–734) and reverse transcriptase (Yamashita et al., 1988, Virol. 163: 112–122). Sequences shown correspond to position 878–930 in the NA gene (Hiti et al., J. Virol. 41: 730–734). The arrows and the underlined nucleotides indicate the changes in the mutant RNA compared to the wild type RNA. Left: Control RNA obtained from influenza A/WSN/33 virus. Right: RNA of mutant virus rescued from MDBK cells which were RNP-transfected with T32NAv mut 2 RNA and infected with helper virus WSN-HK.

FIG. 19. CAT expression in vaccinia virus-infected/ IVACAT-1 RNP transfected cells. Approximately 10^6 mouse C127 cells in 35 mm dishes were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1986) at an M.O.I. of approximately 10 for each vector. After 1.5 hours, synthetic IVACAT-1 RNP was transfected into the virus-infected cells as described (Lutjyes et al., 1989). Cells were incubated overnight, harvested and assayed for CAT activity according to standard procedures (Gorman et al., 1982). The assays contained 0.05 uCl [^{14}C] chloramphenicol, 20 ul of 40 mM acetyl-CoA (Boehringer and 50 ul of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 hours. The labels under the lane numbers indicate the treatment of cells. Lanes 1-control; 2-naked RNA transfection (no polymerase added), no helper virus infection; 3-RNP transfection, no helper virus; 4-RNP transfection, influenza virus as helper; Lanes 5–11-RNP transfection, vaccinia virus vectors as helper viruses express the indicated influenza virus proteins.

FIG. 20. Test of various cell lines. A) Cells were infected with vaccinia vectors expressing the PB2, PB1 and PA proteins (Lanes 1,3,5,7) or the PB2, PB1, PA and NP proteins (Lanes 2,4,6,8), transfected with IVACAT-1 RNP and examined for CAT activity as described. Lanes 1,2: Maden-Darby Canine Kidney (MDCK) cell; 3,4: Hela cells, 5,6: 293 cells (Graham et al., 1977 J. Gen. Virol., 36: 59–72); 7,8 L cells. B) Cell line 3 PNP-4 was used as host cell. Shown under each lane is the influenza viral proteins expressed in each sample. C) 293 cells were infected with the four required vaccinia and transfected with synthetic RNP made using IVA-CAT-I (lane 1) or IVA-CAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assays were performed.

FIG. 21. Schematic representation of the RSV/CAT construct (pRSVA2CAT) used in rescue experiments. The approximate 100 nt long leader and 200 nt long trailer regions of RSV were constructed by the controlled annealing of synthetic oligonucleotides containing partial overlapping complementarity. The overlapping leader oligonucleotides are indicated by the 1L–5L shown in the construct. The overlapping trailer nucleotides are indicated by the 1T–9T shown in the construct. The nucleotide sequences of the leader and trailer oligonucleotides are shown below the construct. The fully formed leader and trailer DNAs were ligated into purified CAT gene DNA at the indicated XbaI and PstI sites respectively. This entire construct was then ligated into KpnI/HindIII digested pUC19. The inclusion of a T7 promoter sequence and a HgaI site flanking the trailer and leader sequences respectively allowed in vitro synthesis of RSV/CAT RNA transcripts containing the precise genomic sequence 3' and 5' ends.

FIG. 22. Thin layer chromatogram (TLC) showing the CAT activity present in 293 cell extracts following infection and transfection with RNA transcribed from the RSV/CAT construct shown in FIG. 1. Confluent monolayers of 293 cells in 6-well plates (~10^6 cells) were infected with either RSV A2 or B9320 at an m.o.i. of 0.1–1.0 pfu cell^{31} ^{1}. At 1 hour post infection the cells were transfected with 5–10 μg of CAT/RSV using the Transfectace™ protocol of Gibco BRL. AT 24 hours post infection the infected/transfected monolayers were harvested and processed for subsequent CAT assay according to Current Protocols in Molecular Biology, Vol. 1, Chapter 9.6.2; Gorman, et al., (1982) Mol. Cell. Biol. 2:1044–1051.

Lanes 1, 2, 3, and 4 show the CAT activity present in (1) uninfected 293 cells, transfected with CAT/RSV RNA, (2) RSV-A2 infected 293 cells, transfected with CAT/RSV RNA, (3) RSV-B9320 infected 293 cells transfected with CAT/RSV RNA, (4) RSV-A2 infected 293 cells, co-infected with supernatant from (2) above. The CAT activity observed in each lane was produced from ⅕ of the total cellular extract from 10^6 cells.

FIG. 23. Schematic representation of the RSV strain A2 genome showing the relative positions of the primer pairs used for the synthesis of cDNAs comprising the entire genome. The endonuclease sites used to splice these clones together are indicated; these sites were present in the native RSV sequence and were included in the primers used for cDNA synthesis. Approximately 100 ng of viral genomic RNA was used in RT/PCR reactions for the separate synthesis of each of the seven cDNAs. The primers for the first and second strand cDNA synthesis from the genomic RNA template are also shown. For each cDNA, the primers for the first strand synthesis are nos. 1–7 and the primers for the second strand sythesis are nos. 1'–7'.

5. DESCRIPTION OF THE INVENTION

This invention relates to the construction and use of recombinant negative strand viral RNA templates which

may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates may be prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or the Sp6 polymerase. Using influenza, for example, the DNA is constructed to encode the message- sense of the heterologous gene sequence flanked upstream of the ATG by the complement of the viral polymerase binding site/promoter of influenza, i.e., the complement of the 3'-terminus of a genome segment of influenza. For rescue in virus particles, it may be preferred to flank the heterologous coding sequence with the complement of both the 3'-terminus and the 5'-terminus of a genome segment of influenza. After transcription with a DNA-directed RNA polymerase, the resulting RNA template will encode the negative polarity of the heterologous gene sequence and will contain the vRNA terminal sequences that enable the viral RNA-directed RNA polymerase to recognize the template.

The recombinant negative sense RNA templates may be mixed with purified viral polymerase complex comprising viral RNA-directed RNA polymerase proteins (the P proteins) and nucleoprotein (NP) which may be isolated from RNP cores prepared from whole virus to form "recombinant RNPs" (rRNPs). These rRNPs are infectious and may be used to express the heterologous gene product in appropriate host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with the rRNPs and virus. Alternatively, the recombinant RNA templates may be used to transfect transformed cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation.

The invention is demonstrated by way of working examples in which RNA transcripts of cloned DNA containing the coding region—in negative sense orientation—of the chloramphenicol acetyltransferase (CAT) gene flanked by the 22 5' terminal and the 26 3' terminal nucleotides of the influenza A/PR/8/34 virus NS RNA were mixed with isolated influenza A virus polymerase proteins. This reconstituted ribonucleoprotein (RNP) complex was transfected into MDCK (or 293) cells, which were infected with influenza virus. CAT activity was negligible before and soon after virus infection, but was demonstrable by seven hours post virus infection. When cell supernatant containing budded virus from this "rescue" experiment was used to infect a new monolayer of MDCK cells, CAT activity was also detected, suggesting that the RNA containing the recombinant CAT gene had been packaged into virus particles. These results demonstrate the successful use of recombinant negative strand viral RNA templates and purified RNA-dependent RNA polymerase to reconstitute recombinant influenza virus RNP. Furthermore, the data suggest that the 22 5' terminal and the 26 3' terminal sequences of the influenza A virus RNA are sufficient to provide the signals for RNA transcription, A replication and for packaging of RNA into influenza virus particles.

Using this methodology we also demonstrated the rescue of synthetic RNAs, derived from appropriate recombinant plasmid DNAs, into stable and infectious influenza viruses. In particular, RNA corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN) was transcribed in vitro from plasmid DNA and, following the addition of purified influenza virus polymerase complex, was transfected into MDBK cells. Superinfection with helper virus lacking the WSN NA gene resulted in the release of virus containing the WSN NA gene. We then introduced five point mutations into the WSN NA gene by cassette mutagenesis of the plasmid DNA. Sequence analysis of the rescued virus revealed that the genome contained all five mutations present in the mutated plasmid. This technology can be used to create viruses with site-specific mutations so that influenza viruses with defined biological properties may be engineered.

The ability to reconstitute RNPs in vitro allows the design of novel chimeric influenza viruses which express foreign genes. One way to achieve this goal involves modifying existing influenza virus genes. For example, the HA gene may be modified to contain foreign sequences in its external domains. Where the heterologous sequence are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a protective immune response against the disease agent from which these determinants are derived. In addition to modifying genes coding for surface proteins, genes coding for nonsurface proteins may be altered. The latter genes have been shown to be associated with most of he important cellular immune responses in the influenza virus system (Townsend et al., 1985, Cell 42: 475–482). Thus, the inclusion of a foreign determinant in the NP or the NS gene of an influenza virus may—following infection—induce an effective cellular immune response against this determinant. Such an approach may be particularly helpful in situations in which protective immunity heavily depends on the induction of cellular immune responses (e.g., malaria, etc.).

Another approach which would permit the expression of foreign proteins (or domains of such proteins) via chimeric influenza viruses concerns the introduction of complete heterologous genes into the virus. Influenza virus preparations with more than eight RNA segments have previously been described (Nayak, D. et al. in Genetics of Influenza Virus, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255–279). Thus, chimeric influenza viruses with nine or more RNA segments may be viable, and correct packaging of such chimeric viruses may readily occur.

The invention may be divided into the following stages solely for the purpose of description and not by way of limitation: (a) construction of recombinant RNA templates; b) expression of heterologous gene products using the recombinant RNA templates; and (c) rescue of the heterologous gene in recombinant virus particles. For clarity of discussion, the invention is described in the subsections below using influenza. Any strain of influenza (e.g., A, B, C) may be utilized. However, the principles may be analogously applied to construct other negative strand RNA virus templates and chimeric viruses including, but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus; bunyaviruses; arena viruses; etc. Section 9 describes and exemplifies the application of these principles to the construction of respiratory syncytial virus templates and chimeric respiratory syncytial viruses. A particularly interesting virus system that can be used in accordance with the invention are the orthomyxo-like insect virus called Dhori (Fuler, 1987, Virology 160: 81–87).

5.1. Construction of the Recombinant RNA Templates

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g, the complement of 3'-influenza virus terminus, or the complements of both the 3'-and 5'-influenza virus termini may be constructed using techniques known in the art. Recombinant DNA molecules containing these hybrid

sequences can be cloned and transcribed by a DNA-directed RNA polymerase, such as bacteriophage T7, T3 or the Sp6 polymerase and the like, to produce the recombinant RNA templates which possess the appropriate viral sequences that allow for viral polymerase recognition and activity.

One approach for constructing these hybrid molecules is to insert the heterologous coding sequence into a DNA complement of an influenza virus genomic segment so that the heterologous sequence is flanked by the viral sequences required for viral polymerase activity; i.e., the viral polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding site. In an alternative approach, oligonucleotides encoding the viral polymerase binding site, e.g., the complement of the 3'-terminus or both termini of the virus genomic segments can be ligated to the heterologous coding sequence to construct the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a target sequence was formerly dictated by the presence of appropriate restriction enzyme sites within the target sequence. However, recent advances in molecular biology have lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere within a target sequence through the use of site-directed mutagenesis (e.g., see, for example, the techniques described by Kunkel, 1985, Proc. Natl. Acad. Sci. U.S.A. 82;488). Variations in polymerase chain reaction (PCR) technology, described infra, also allow for the specific insertion of sequences (i.e., restriction enzyme sites) and allow for the facile construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA polymerase promoter (e.g., bacteriophage T3, T7 or Sp6) and the hybrid sequence containing the heterologous gene and the influenza viral polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the vital polymerase binding site using an RNA ligase. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections below.

5.1.1. The Viral 3'-Terminus is Required for Polymerase Activity

The experiments described in Section 6 et seq., infra, are the first to define promoter sequences for a polymerase of a negative-sense RNA virus, and it was found that the specificity lies in the 3' terminal 15 nucleotides. These viral polymerase binding site sequences, as well as functionally equivalent sequences may be used in accordance with the invention. For example, functionally equivalent sequences containing substitutions, insertions, deletions, additions or inversions which exhibit similar activity may be utilized. The RNA synthesis by the viral polymerase described infra is a model for specific recognition and elongation by the influenza viral polymerase for the following reasons: (a) the polymerase has high activity when primed with ApG, a feature unique to influenza viral polymerase; (b) it has optimal activity at temperature and ionic conditions previously shown to be effective for the viral RNPs; (c) the polymerase is specific for influenza viral sequences on the model RNA templates; (d) the polymerase is active in the cap-endonuclease primed RNA synthesis which is the hallmark of the influenza viral polymerase; (e) recognition of cap donor RNA is specific to cap 1 structures; and (f) genomic RNA segments are specifically copied.

5.1.2. Terminal Panhandle is not Required for Optimal Recognition and Synthesis by the Viral Polymerase

We had previously shown that the influenza vital segment RNAs base-pair at their termini to form panhandle structures. This was achieved by two methods. A cross-linking reagent derivative of psoralen covalently bound the termini of each segment in intact virus or in RNPs from infected cells (Hsu et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140–8144). The treated RNA was seen by electron microscopy to be circular, by virtue of the cross-linked termini. Similarly, the RNA termini in RNPs were found to be sensitive to ribonuclease V1, which recognizes and cleaves double-stranded RNA, and the viral polymerase was found to be bound to both termini in the panhandle conformation (Honda, et al., 1988, J. Biochem. 104: 1021–1026). In these studies the panhandle structure of the genomic RNA was shown to exist, and it was inferred to play a role in polymerase recognition. Although the template RNAs used in the examples described, were originally prepared to reveal panhandle-specific protein binding, it was found that the terminal panhandle had no obvious role in the polymerase reactions studied herein.

5.1.3. The RNA Polymerase Preparation Specifically Copies Negative Sense Templates

The viral polymerase was shown to synthesize RNA with optimal efficiency if the template had the "wild-type" negative sense 3' terminus. It was shown that RNAs of unrelated sequence were not copied, and that those with extra polylinker sequences on the 3' end were much less efficiently copied. A DNA of the correct sequence was similarly unsuitable as a template. The reaction was highly specific since the M-wt template was replicated only at very low levels. Even though our source of polymerase was intact virus, this finding was very surprising since it had never been suggested that the polymerase which recognizes the viral sense RNA would not efficiently copy the plus sense strand. Studies are underway to examine the specificity of the polymerase purified from infected cells at times post infection when the complementary RNA is copied into genomic templates. The present data support a model whereby the viral polymerase which copies vRNA is functionally different from that which synthesizes vRNA from cRNA by virtue of their promoter recognition. It is possible that by regulated modification of the polymerase in infected cells it then becomes capable of recognizing the 3' terminus of plus sense RNA. By analyzing promoter mutants we investigated the fine specificity of the reaction and found that the only single mutation which generated a significantly lower level of synthesis was that of V-A_3 RNA. Furthermore, combinations of two or more point changes in positions 3, 5, 8 and 10 greatly lowered synthesis levels.

5.1.4. Insertion of the Heterologous Gene Sequence into the PB2 PB1 PA or NP Gene Segments

The gene segments coding for the PB2, PB1, PA and NP proteins contain a single open reading frame with 24–45 untranslated nucleotides at their 5'-end, and 22–57 untranslated nucleotides at their 3'-end. Insertion of a foreign gene sequence into any of these segments could be accomplished by either a complete replacement of the viral coding region with the foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis. The principle of this mutagenesis method is illustrated in FIG. **10**. Briefly, PCR-primer A would contain, from 5' to 3', a unique restriction enzyme site, such as a class IIS restriction enzyme site (i.e., a "shifter" enzyme; that recognizes a specific sequence but

cleaves the DNA either upstream or downstream of that sequence); the entire 3' untranslated region of the influenza gene segment; and a stretch of nucleotides complementary to the carboxy-terminus coding portion of the foreign gene product. PCR-primer B would contain from the 5' to 3' end: a unique restriction enzyme site; a truncated but active phage polymerase sequence; the complement of the entire 5' untranslated region of the influenza gene segment (with respect to the negative sense vRNA); and a stretch of nucleotides corresponding to the 5' coding portion of the foreign gene. After a PCR reaction using these primers with a cloned copy of the foreign gene, the product may be excised and cloned using the unique restriction sites. Digestion with the class IIS enzyme and transcription with the purified phage polymerase would generate an RNA molecule containing the exact untranslated ends of the influenza viral gene segment with a foreign gene insertion. Such a construction is described for the chloramphenicol acetyltransferase (CAT) gene used in the examples described in Section 7 infra. In an alternate embodiment, PCR-primed reactions could be used to prepare double-stranded DNA containing the bacteriophage promoter sequence, and the hybrid gene sequence so that RNA templates an be transcribed directly without cloning.

Depending on the integrity of the foreign gene product and the purpose of the construction, it may be desirable to construct hybrid sequences that will direct the expression of fusion proteins. For example, the four influenza virus proteins, PB2, PB1, PA or NP are polymerase proteins which are directed to the nucleus of the infected cell through specific sequences present in the protein. For the NP this amino acid sequence has been found to be (single letter code) QLVWMACNSAAFEDLRVLS (Davey et al., 1985, Cell 40: 667–675). Therefore, if it is desired to direct the foreign gene product to the nucleus (if by itself it would not ordinarily do so) the hybrid protein should be engineered to contain a domain which directs it there. This domain could be of influenza viral origin, but not necessarily so. Hybrid proteins can also be made from non-viral sources, as long as they contain the necessary sequences for replication by influenza virus (3' untranslated region, etc.). As another example, certain antigenic regions of the viral gene products may be substituted with foreign sequences. Townsend et al., (1985, Cell 42: 475–482), identified an epitope within the NP molecule which is able to elicit a vigorous CTL (cytotoxic T cell) response. This epitope spans residues 147–161 of the NP protein and consists of the amino acids TYQRTRQLVRLTGMDP. Substituting a short foreign epitope in place of this NP sequence may elicit a strong cellular immune response against the intact foreign antigen. Conversely, expression of a foreign gene product containing this 15 amino acid region may also help induce a strong cellular immune response against the foreign protein.

5.1.5. Insertion of the Heterologous Gene Sequence into the HA or NA Gene Segments

The HA and NA proteins, coded for by separate gene segments, are the major surface glycoproteins of the virus. Consequently, these proteins are the major targets for the humoral immune response after infection. They have been the most widely-studied of all the influenza viral proteins as the three-dimensional structures of both these proteins have been solved.

The three-dimensional structure of the H3 hemagglutinin along with sequence information on large numbers of variants has allowed for the elucidation of the antigenic sites on the HA molecule (Webster et al., 1983, In Genetics Of Influenza Virus, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 127–160). These sites fall into four discrete non-overlapping regions on the surface of the HA. These regions are highly variable and have also been shown to be able to accept insertions and deletions. Therefore, substitution of these sites within HA (e.g., site A; amino acids 122–147 of the A/HK/68 HA) with a portion of a foreign protein may provide for a vigorous humoral response against this foreign peptide. In a different approach, the foreign peptide sequence may be inserted within the antigenic site without deleting any viral sequences. Expression products of such constructs may be useful in vaccines against the foreign antigen, and may indeed circumvent a problem discussed earlier, that of propagation of the recombinant virus in the vaccinated host. An intact HA molecule with a substitution only in antigenic sites may allow for HA function and thus allow for the construction of a viable virus. Therefore, this virus can be grown without the need for additional helper functions. Of course, the virus should be attenuated in other ways to avoid any danger of accidental escape.

Other hybrid constructions may be made to express proteins on the cell surface or enable them to be released from the cell. As a surface glycoprotein, the HA has an amino-terminal cleavable signal sequence necessary for transport to the cell surface, and a carboxy-terminal sequence necessary for membrane anchoring. In order to express an intact foreign protein on the cell surface it may be necessary to use these HA signals to create a hybrid protein. Alternatively, if only the transport signals are present and the membrane anchoring domain is absent, the protein may be excreted out of the cell.

In the case of the NA protein, the three-dimensional structure is known but the antigenic sites are spread out over the surface of the molecule and are overlapping. This indicates that if a sequence is inserted within the NA molecule and it is expressed on the outside surface of the NA it will be immunogenic. Additionally, as a surface glycoprotein, the NA exhibits two striking differences from the HA protein. Firstly, the NA does not contain a cleavable signal sequence; in fact, the amino-terminal signal sequence acts as a membrane anchoring domain. The consequence of this, and the second difference between the NA and HA, is that the NA is orientated with the amino-terminus in the membrane while the HA is orientated with the carboxy-terminus in the membrane. Therefore it may be advantageous in some cases to construct a hybrid NA protein, since the fusion protein will be orientated opposite of a HA-fusion hybrid.

5.1.6. Insertion of the Heterologous Gene into the NS and M Gene Segments

The unique property of the NS and M segments as compared to the other six gene segments of influenza virus is that these segments code for at least two protein products. In each case, one protein is coded for by an mRNA which is co-linear with genomic RNA while the other protein is coded for by a spliced message. However, since the splice donor site occurs within the coding region for the co-linear transcript, the NS1 and NS2 proteins have an identical 10 amino acid amino terminus while MI and M2 have an identical 14 amino acid amino terminus.

As a result of this unique structure, recombinant viruses may be constructed so as to replace one gene product within the segment while leaving the second product intact. For instance, replacement of the bulk of the NS2 or M2 coding region with a foreign gene product (keeping the splice acceptor site) could result in the expression of an intact NS1

or M1 protein and a fusion protein instead of NS2 or M2. Alternatively, a foreign gene may be inserted within the NS gene segment without affecting either NS1 or NS2 expression. Although most NS genes contain a substantial overlap of NS1 and NS2 reading frames, certain natural NS genes do not. We have analyzed the NS gene segment from A/Ty/Or/71 virus (Norton et al., 1987, Virology 156: 204–213) and found that in this particular gene, the NS1 protein terminates at nucleotide position 409 of the NS gene segment while the splice acceptor site for the NS2 is at nucleotide position 528. Therefore, a foreign gene could be placed between the termination codon of the NS1 coding region and the splice acceptor site of the NS2 coding region without affecting either protein. It may be necessary to include a splice acceptor site at the 5' end of the foreign gene sequence to ensure protein production (this would encode a hybrid protein containing the amino-terminus of NS1). In this way, the recombinant virus should not be defective and should be able to be propagated without need of helper functions.

Although the influenza virus genome consists of eight functional gene segments it is unknown how many actual segments a virus packages. It has been suggested that influenza can package more than eight segments, and possibly up to 12 (Lamb and Choppin, 1983, Ann. Rev. Biochem. 52: 467–506). This would allow for easier propagation of recombinant virus in that "ninth" gene segment could be designed to express the foreign gene product. Although this "ninth" segment may be incorporated into some viruses, it would soon be lost during virus growth unless some selection is supplied. This can be accomplished by "uncoupling" the NS or M gene segment. The NS2 coding portion could be removed from the NS gene segment and placed on the gene segment coding for the foreign protein (along with appropriate splicing signals). Alternatively, a bicistronic mRNA could be constructed to permit internal initiation to "unsplice" these viral sequences; for example, using the sequences described by Pelletier et al., 1988, Nature 334: 320–325. The resulting recombinant virus with the "uncoupled" NS or M gene would be able to propagate on its own and also would necessarily have to package the "ninth" gene segment, thus ensuring expression of the foreign gene.

5.2. Expression of Heterologous Gene Products Using Recombinant RNA Template

The recombinant templates prepared as described above can be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant template can be combined with viral polymerase complex purified as described in Section 6, infra, to produce rRNPs which are infectious. Alternatively, the recombinant template may be mixed with viral polymerase complex prepared using recombinant DNA methods (e.g., see Kingsbury et al., 1987, Virology 156: 396–403). Such rRNPs, when used to transfect appropriate host cells, may direct the expression of the heterologous gene product at high levels. Host cell systems which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with influenza, cell lines engineered to complement influenza viral functions, etc.

In an alternate embodiment of the invention, the recombinant templates or the rRNPs may be used to transfect cell lines that express the viral polymerase proteins in order to achieve expression of the heterologous gene product. To this end, transformed cell lines that express all three polymerase proteins such as 3P-38 and 3P-133 (Krystal et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83: 2709–2713) may be utilized as appropriate host cells. Host cells may be similarly engineered to provide other viral functions or additional functions such as NP.

5.2.1. Purification of the Viral Polymerase

The viral polymerase proteins used to produce the rRNPs may be purified from dissociated RNP cores isolated from whole virus. In general, RNP cores may be prepared using standard methods (Plotch et al., 1981, Cell 23: 847–858; Rochavansky, 1976, Virology 73: 327–338). The pooled RNP cores may then be centrifuged on a second gradient of CsCl (1.5–3.0M) and glycerol (30%–45%) as described by Honda et al., 1988, J. Biochem. 104: 1021–1026. The active viral polymerase fractions may be isolated from top of the gradient, i.e., in the region of the gradient correlating with 1.5 to 2.0M CsCl and corresponding to the fraction Honda et al. identified as "NP". Surprisingly, this fraction contains all the viral polymerase proteins required for the active complex. Moreover, the P proteins which may be recovered from the bottom of the gradient are not required, and indeed do not provide for the transcription of full length viral RNA. Thus, it appears that the so-called "NP" fraction contains, in addition to NP, the active forms of the PB2, PB1, and PA proteins.

5.2.2. High Concentrations of Polymerase are Required for Cap-Primed RNA Synthesis

High concentrations of viral polymerase complex are able to catalyze this virus-specific cap-endonuclease primed transcription. Under the conditions specified in Section 6 infra, about 50 ng NP with 200 pg of the three P proteins were found to react optimally with 5 to 10 ng RNA reaction. The observation has been that although the NP selectively encapsidates influenza vRNA or cRNA in vivo, the NP will bind to RNA nonspecifically in vitro (Kingsbury, et al., 1987, Virology 156: 396–403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11–16). Presumably, in order for the viral polymerase to recognize the viral template RNAs in our in vitro reaction, they have to be encapsidated by the NP. Therefore, the addition of a capped mRNA primer would essentially compete with the template RNA for binding of NP. Since the dinucleotide ApG would not be expected to bind NP, the low concentration polymerase was able to use only the short templates with ApG. Supporting this hypothesis is the observation that the higher concentration polymerase preparation is inhibited through the addition of progressively higher amounts of either template RNA or any non-specific RNA. It should also be noted that the unusual specificity for the m7GpppXm cap 1 structure previously shown with viral RNPs was also found with the reconstituted RNPs.

5.2.3. Genomic Length RNA Templates are Efficiently Copied

Plasmid-derived RNA identical to segment 8 of the A/WSN/33 virus was specifically copied by the polymerase (using the PCR method described in FIG. 10). In reactions using RNA extracted from virus, all eight segments were copied, although the HA gene was copied at a lower level. The background in these reactions was decreased in comparison to the 30 to 53 nt templates, probably since the contaminating RNAs in the polymerase preparation were predominantly defective RNAs of small size. Recombinant templates encoding foreign genes transcribed in this system may be used to rescue the engineered gene in a virus particle.

5.3. Preparation of Chimeric Negative Strand RNA Virus

In order to prepare chimeric virus, reconstituted RNPs containing modified influenza virus RNAs or RNA coding

for foreign proteins may be used to transfect cells which are also infected with a "parent" influenza virus. Alternatively, the reconstituted RNP preparations may be mixed with the RNPs of wild type parent virus and used for transfection directly. Following reassortment, the novel viruses may be isolated and their genomes be identified through hybridization analysis. In additional approaches described herein for the production of infectious chimeric virus, rRNPs may be replicated in host cell systems that express the influenza viral polymerase proteins (e.g., in virus/host cell expression systems; transformed cell lines engineered to express the polymerase proteins, etc.), so that infectious chimeric virus are rescued; in this instance, helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed. In a particularly desirable approach, cells infected with rRNPs engineered for all eight influenza virus segments may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system.

Theoretically, one can replace any one of the eight gene segments, or part of any one of the eight segments with the foreign sequence. However, a necessary part of this equation is the ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem. We have shown that mutants of influenza virus defective in the PB2 and NP proteins can be grown to substantially higher titers in cell lines which were constructed to constitutively express the polymerase and NP proteins (Krystal et al., 1986 Proc. Natl. Acad. Sci. U.S.A. 83: 2709–2813). Similar techniques may be used to construct transformed cell lines that constitutively express any of the influenza genes. These cell lines which are made to express the viral protein may be used to complement the defect in the recombinant virus and thereby propagate it. Alternatively, certain natural host range systems may be available to propagate recombinant virus. An example of this approach concerns the natural influenza isolate CR43-3. This virus will grow normally when passaged in primary chick kidney cells (PCK) but will not grow in Madin-Darby canine kidney cells (MDCK), a natural host for influenza (Maassab & DeBorde, 1983, Virology 130: 342–350). When we analyzed this virus we found that it codes for a defective NS1 protein caused by a deletion of 12 amino acids. The PCK cells contain some activity which either complements the defective NS1 protein or can completely substitute for the defective protein.

A third approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. This would be an analogous situation to the propagation of defective-interfering particles of influenza virus (Nayak et al., 1983, In: Genetics of Influenza Viruses, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255–279). In the case of defective-interfering viruses, conditions can be modified such that the majority of the propagated virus is the defective particle rather than the wild-type virus. Therefore this approach may be useful in generating high titer stocks of recombinant virus. However, these stocks would necessarily contain some wild-type virus.

Alternatively, synthetic RNPs may be replicated in cells co-infected with recombinant viruses that express the influenza virus polymerase proteins. In fact, this method may be used to rescue recombinant infectious virus in accordance with the invention. To this end, the influenza virus polymerase proteins may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus, adenovirus, baculovirus, etc.) or cell lines that express the polymerase proteins (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709–2713). Moreover, infection of host cells with rRNPs encoding all eight influenza virus proteins may result in the production of infectious chimeric virus particles. This system would eliminate the need for a selection system, as all recombinant virus produced would be of the desired genotype. In the examples herein, we describe a completely synthetic replication system where, rather than infecting cells with influenza virus, synthetic RNP's are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia vectors. In this way we show that the only influenza virus proteins essential for transcription and replication of RNP are the three polymerase proteins and the nucleoprotein.

It should be noted that it may be possible to construct a recombinant virus without altering virus viability. These altered viruses would then be growth competent and would not need helper functions to replicate. For example, alterations in the hemagglutinin gene segment and the NS gene segment discussed, supra, may be used to construct such viable chimeric viruses.

In the examples infra, the construction of a recombinant plasmid is described that, following transcription by T7 polymerase, yielded an RNA template which was recognized and transcribed by the influenza virus polymerase in vitro. This RNA template corresponds to the NS RNA of an influenza virus except that the viral coding sequences are replaced by those of a CAT gene. This recombinant negative strand viral RNA template was then mixed with purified influenza virus polymerase to reconstitute an RNP complex. The recombinant RNP complex was transfected into cells which were then infected with influenza virus, leading to expression of CAT activity.

A number of factors indicate that this system represents a biologically active recombinant RNP complex which is under tight control of the signals for transcription, replication and packaging of influenza virus RNAs. First, the CAT gene is of negative polarity in the recombinant viral RNA used for RNP transfection. Thus, the incoming RNA cannot be translated directly in the cell and must first be transcribed by the influenza virus polymerase to permit translation and expression of the CAT gene. Secondly, neither transfected naked recombinant RNA alone in the presence of infecting helper virus, nor recombinant RNP complex in the absence of infecting helper virus is successful in inducing CAT activity. This suggests that influenza viral proteins provided by the incoming RNP, as well as by the infecting helper virus, are necessary for the amplification of the recombinant RNA template. Finally, after RNP-transfection and infection by helper virus, virus particles emerge which apparently contain the recombinant RNA, since these particles again induce CAT activity in freshly infected cells. These results suggest that the 26 3' terminal and the 22 5' terminal nucleotides corresponding to the terminal nucleotides in the influenza A virus NS RNA are sufficient to provide the signals for polymerase transcription and replication, as well as for packaging of the RNA into particles.

The foregoing results, which defined the cis acting sequences required for transcription, replication and packaging of influenza virus RNAs, were extended by additional working examples, described infra, which demonstrate that

recombinant DNA techniques can be used to introduce site-specific mutations into the genomes of infectious influenza viruses.

Synthetic RNAs, derived by transcription of plasmid RNA in vitro were used in RNP-transfection experiments to rescue infectious influenza virus. To enable selection of this virus, we chose a system that required the presence of a WSN-like neuraminidase gene in the rescued virus. Viruses containing this gene can grow in MDBK cells in the absence of protease in the medium (Schulman et al., 1977, J. Virol. 24: 170–176). The helper virus WSN-HK does not grow under these circumstances. Clearly, alternative selection systems exist. For example, antibody screens or conditionally lethal mutants could be used to isolate rescued viruses containing RNAs derived from plasmid DNAs. In the experiments viruses described infra, viruses which were WSN virus-like were recovered. The WSN NA gene was derived from plasmid DNAs or from purified WSN virion RNA (FIG. **17**, lanes 2 and 5). In the latter case, using whole virion RNA for the RNP-transfection, we do not know whether other genes were also transferred to the rescued virus, since the helper virus shares the remaining seven genes with WSN virus. The rescued viruses had the expected RNA patterns (FIG. **17**) and grew to titers in MDBK or MDCK cells which were indistinguishable from those of the wild type WSN virus. It should be noted that rescue of an NA RNA containing a single nucleotide deletion in the 5' nontranslated region was not possible. This again illustrates the importance of regulatory sequences present in the nontranslated regions of influenza virus RNAs. We also rescued virus using RNA that was engineered to contain 5 nucleotide changes in a 39 nucleotide long region (FIG. **16**). We verified the presence of these mutations in the rescued mutant virus by direct sequencing of the RNA (FIG. **18**). These mutations did not result in any amino acid change in the neuraminidase protein and thus were not expected to change the biological property of the virus. Although this virus was not extensively studied, its plaquing behavior and its growth characteristics were indistinguishable from that of wild type WSN virus. Using such technology, mutations may be introduced that will change the biological characteristics of influenza viruses. These studies will help in distinguishing the precise functions of all the viral proteins, including those of the nonstructural proteins. In addition, the nontranslated regions of the genome can be studied by mutagenesis, which should lead to a better understanding of the regulatory signals present in viral RNAs. An additional area of great interest concerns the development of the influenza virus system as a vaccine vector.

5.4. Vaccine Formulations Using the Chimeric Viruses

Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g., gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric viruses of the invention.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

In this regard, the use of genetically engineered influenza virus (vectors) for vaccine purposes may require the presence of attenuation characteristics in these strains. Current live virus vaccine candidates for use in humans are either cold adapted, temperature sensitive, or passaged so that they derive several (six) genes from avian viruses, which results in attenuation. The introduction of appropriate mutations (e.g., deletions) into the templates used for transfection may produce the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaptation can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low.

Alternatively, chimeric viruses with "suicide" characteristics may be constructed. Such viruses would go through only one or a few rounds of replication in the host. For example, cleavage of the HA is necessary to allow for reinitiation of replication. Therefore, changes in the HA cleavage site may provide a virus that replicates in an appropriate cell system but not in the human host. When used as a vaccine, the recombinant virus would go through a single replication cycle and induce a sufficient level of immune response but it would not go further in the human host and cause disease. Recombinant viruses lacking one or more of the essential influenza virus genes would not be able to undergo successive rounds of replication. Such defective viruses can be produced by co-transfecting reconstituted RNPs lacking a specific gene(s) into cell lines which permanently express this gene(s). Viruses lacking an essential gene(s) will be replicated in these cell lines but when administered to the human host will not be able to complete a round of replication. Such preparations may transcribe and translate—in this abortive cycle—a sufficient number of genes to induce an immune response. Alternatively, larger quantities of the strains could be administered, so that these preparations serve as inactivated (killed) virus vaccines. For inactivated vaccines, it is preferred that the heterologous gene product be expressed as a viral component, so that the gene product is associated with the virion. The advantage of such preparations is that they contain native proteins and do not undergo inactivation by treatment with formalin or other agents used in the manufacturing of killed virus vaccines.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β-propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.

Inactivated viruses may be formulated with a suitable adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG and Corynebacterium parvum.

Many methods may be used to introduce the vaccine formulations described above. These include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed. Where a live chimeric virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus. The ability of influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by chimeric influenza viruses may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against a particular disease causing agent.

6. EXAMPLE: PROMOTER ANALYSIS OF THE INFLUENZA VIRAL RNA POLYMERASE

In the examples described below, polymerase which is depleted of genomic RNA was prepared from the upper fractions of the CsCl-glycerol gradient centrifugation. This polymerase is able to copy short model templates which are derived from transcription of appropriate plasmic DNA with bacteriophage T7 RNA polymerase in a sequence-specific manner. The termini of this model RNA are identical to the 3' 15 and 5' 22 nucleotides conserved in segment 8 from all influenza A viral RNAs. By manipulating the plasmid in order to prepare different RNAs to serve as template, we demonstrated that recognition of and synthesis from this model RNA was specific for the promoter at the 3' terminal sequence and did not require the panhandle. In addition, site specific mutagenesis identified nucleotide positions responsible for the viral polymerase favoring synthesis from genomic sense templates over complementary sense RNA. Conditions were also found in which cap-endonuclease primed RNA synthesis could be observed using model RNAs. In addition, the reconstituted system permitted virus-specific synthesis from genomic length RNAs, derived either from plasmids or from RNA purified from virus through phenol extraction.

6.1. Materials and Methods

6.1.1. Purification of the Viral RNA Polymerase

RNP cores were prepared from whole virus using standard methods (Plotch, et al., 1981, Cell 23: 847–858; Rochavansky, 1976, Virology 73: 327–338). Two to three milligrams of virus were disrupted by incubating in 1.5% Triton N-101, 10 mg/ml lysolecithin, 100 mM tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and 1.5 mM dithiothreitol. The sample was fractionated by centrifugation on a 30–70% glycerol (w/v) step gradient in the presence of 50 mM tris-HCl, pH 7.8 and 150 mM NaCl. The core preparation was centrifuged at 45,000 rpm in an SW50.1 rotor for 4 hours at 4° C. Fractions enriched in RNP were identified by SDS-polyacrylamide gel electrophoresis of protein samples from each fraction and staining with silver. The core fractions were then subjected to a second gradient centrifugation as was described in Honda et al. 1988, J. Biochem. 104: 1021–1026. This second gradient had steps of 0.5 ml 3.0M CsCl and 45% (w/v) glycerol, 1.75 ml 2.5 M CsCl and 40% glycerol, 1.25 ml 2.0M CsCl and 35% glycerol, and 1.0 ml of 1.5 M CsCl and 30% glycerol. All steps were buffered with 50 mM tris-HCl, pH 7.6 and 100 mM NaCl. 0.5 ml of RNP cores were layered on top and the sample was centrifuged at 45,000 rpm in an SW50.1 rotor for 25 hours at 4° C. Polymerase fractions were again identified by SDS-polyacrylamide electrophoresis of the protein samples and silver staining. Active polymerase fractions were generally found in the region of the gradient correlating with 1.5 to 2.0 M CsCl. These fractions were pooled and then dialyzed against 50 mM tri-HCl, pH 7.6, 100 mM NaCl and 10 mM $MgCl_2$, and concentrated in centricon-10 tubes (Amicon) or fractions were dialyzed in bags against 50 mM tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 50% glycerol.

6.1.2. Preparation of Plasmid

The plasmid design is indicated in FIG. 2. Insert DNA for the pV-wt plasmid was prepared using an Applied Biosystems DNA synthesizer. The "top" strand was

5'- GAAGCTTAATACGACTCACTATAAGTAGAAACAAGGGTG
TTTTTTCATATCATTTAAACTTCACCCTGCTTTTGCTGAA
TTCATTCTTCTGCAGG-3'.

The "bottom" strand was synthesized by primer-extension with

5'- CCTGCAGAAGAATGA-3'

as primer. The 95 bp DNA was digested with HindIII and PstI and purified by extraction with phenol/chloroform, ethanol precipitation, and passage over a NACS-prepack ion Exchange column (Bethesda Research Laboratories). This DNA was ligated into pUC-19 which had been digested with HindIII and PstI and then used to transform *E. coli* strain DH5-α which had been made competent using standard protocols. Bacteria were spread on agar plates containing X-gal and IPTG, and blue colonies were found to have the plasmid containing the predicted insert since the small insert conserved the lacZ reading frame and did not contain a termination codon. The pM-wt plasmid was prepared by a similar strategy except that both strands were chemically synthesized with the upper strand having the sequence

5'- GAAGCTTAATACGACTCACTATAAGCAAAAGCAGGGTGA
AGTTTAAATGATAT — GAAAAAACACCCTTGTTTCTACTG
AATTCATTCTTCTGCAGG-3'.

The pV-d5' plasmid (FIG. 2) was prepared using the oligonucleotides

5'- AGCTTAATACGACTCACTATAAGATCTATTAAACT — T
CACCCTGCTTTTGCTGAATTCATTCTTCTGA-3' and

5'- GAAGAATGAAT — TCAGCAAAAGCAGGGTGAAGTTTAA
TAGATCTTATAGTGAGTCGTATTA-3'.

The DNAs were annealed and ligated into the HindIII/PstI digested pUC-19 and white colonies were found to contain the correct plasmid because this insert resulted in a frameshift in the lacZ gene. The point mutants were isolated following digestion of pV-d5' with BglII and PstI and ligation of the linearized plasmid with a single stranded oligonucleotide of mixed composition. Since BglII laves a 5' extension and PstI a 3' extension, a single oligonucleotide was all that was necessary for ligation of insert. The host cell was then able to repair gaps caused by the lack of a

complementary oligonucleotide. Oligonucleotides were designed to repair the frameshift in the lacZ gene so that bacteria which contained mutant plasmids were selected by their blue color.

Plasmid pHgaNS, which was used to prepare an RNA identical to segment 8 of A/WSN/33, was prepared using the primers

5'- CCGAATTCTTAATACGACTCACTATAAGTAGAAACAAGG GTG-3' and

5'- CCTCTAGACGCTCGAGAGCAAAAGCAGGTG-3'

in a polymerase chain reaction off of a cDNA clone. The product was then cloned into the XbaI/EcoRI window of pUC19.

6.1.3. Preparation of RNA Templates

Plasmid DNAs were digested with MboII or other appropriate endonucleases (see FIG. 2), and the linearized DNA was transcribed using the bacteriophage T7 RNA polymerase. Run-off RNA transcripts were treated with RNAse-free DNAse 1 and then the RNA was purified from the proteins and free nucleotides using Qiagen tip-5 ion exchange columns (Qiagen, Inc.). Following precipitation in ethanol, purified RNAs were resuspended in water and a sample was analyzed by electrophoresis and followed by silver staining of the polyacrylamide gel in order to quantify the yield of RNA.

6.1.4. Influenza Viral Polymerase Reactions

In a 25 μl total volume, about 30 μg of nucleoprotein and 200 pg total of the three polymerase proteins were mixed with 10 ng of template RNA and the solution was made up to a final concentration of: 50 mM Hepes pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% NP-40, 0.4 mM adenylyl-(3'–5')-guanosyl (ApG) dinucleotide (Pharmacia), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and approximately 0.6 μM α-^{32}P-UTP (40 μCi at 3000 Ci/mmole, New England Nuclear). Reactions were assembled on ice and then transferred to a 30° C. water bath for 90 minutes. Reactions were terminated by the addition of 0.18 ml ice-cold 0.3M sodium acetate/10 mM EDTA and were then extracted with phenol/chloroform (1:1 volume ratio). Following the first extraction, 15 μg polyI-polyC RNA was added as carrier, and the sample was extracted again with phenol/ chloroform. The samples were then extracted with ether and precipitated in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and then dried under vacuum.

In reactions using the high concentration polymerase, conditions were identical as above except that 20 ng of template RNA were added. In reactions using genomic length RNAs, the amount of polymerase used was doubled, 50 ng of template RNA was used, and the UTP concentration was raised to 2.6 μM.

The RNA was resuspended in a dye mix containing 78% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.05% bromophenol blue. Typically, a sample from this RNA was electrophoresed on an 8% polyacrylamide gel in the absence of urea, and the remainder was denatured by heating to 100° C. for 1.5 minutes and an aliquot was loaded on an 8% polyacrylamide gel containing 7.7M urea. Gels were fixed by a two step procedure, first in 10% acetic acid, and then in 25% methanol/8% acetic acid. Gels were dried onto filter paper and then exposed to x-ray film.

When different RNAs were being tested for use as template, the different RNA preparations were always analyzed on polyacrylamide gels and stained with silver in order that equal amounts of each template were used. To quantitate the amount of product, gels were exposed to x-ray film in the absence of an intensifying screen in order to improve the linearity of the densitometer readings. Autoradiographs were analyzed using a FB910 scanning densitometer (Fisher Biotech) and peaks were evaluated using computer software from Fisher Biotech.

6.1.5. Nuclease Analysis of Reaction Products

For ribonuclease T1 analysis of the two principle RNA products, reaction products were analyzed by 8% polyacrylamide gel electrophoresis (without urea) and the gel was not treated with fixative. The wet gel was exposed to an x-ray film and the appropriate gel pieces were located and excised. The gel piece was crushed in 0.3 ml containing 10 mM tris pH 7.5, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 1 μg tRNA as carrier. The RNA diffused into this solution for 3 hours and then the gel was pelleted and the supernatant was made 0.3M in sodium acetate. The supernatant was then extracted twice in phenol/chloroform and once in ether and then precipitated in ethanol. The RNA pellet was resuspended in 5 μl formamide, denatured in boiling water for 1.5 minutes and then diluted by the addition of 0.1 ml 10 mM tris-HCl, pH 7.5, and 1 mM EDTA. Ribonuclease T1 (50 units, Boehringer Mannheim Biochemicals) was added and the samples were incubated for 60 minutes at 37° C. V-wt and M-wt RNAs synthesized with T7 RNA polymerase in the presence of α-^{32}P- UTP were similarly digested with RNAse T1. Reaction products were extracted in phenol/ chloroform and precipitated in ethanol and then were analyzed on 20% polyacrylamide gels containing 7.7M urea.

Nuclease S1 analysis of reaction products was done on transcribed RNA by first terminating the standard polymerase reaction through the addition of SI buffer to a volume of 0.2 ml with 0.26M NaCl, 0.05M sodium acetate, pH 4.6, and 4.5 mM zinc sulfate. The sample was divided into two 0.1 ml volumes and 100 units of S1 nuclease (Sigma Chemical Company) were added to one tube. The samples were incubated for 60 minutes at 37° C. Following the incubation, EDTA (10 mM final concentration) and 15 μg polyI-polyC RNA was added and the sample was extracted with phenol/chloroform and precipitated in ethanol. The samples were then subjected to polyacrylamide gel electrophoresis.

6.2. Results

6.2. 1. Preparation of Influenza Viral RNA Polymerase and of Template RNA

RNP cores of influenza virus A/Puerto Rico/8/34 were prepared by disruption of virus in lysolecithin and Triton N-101 followed by glycerol gradient centrifugation (Rochavansky, 1976, Virology 73: 327–338). Fractions containing cores were then subjected to a second centrifugation in a CsCl-glycerol step gradient (Honda, et al., 1988, J. Biochem. 104: 1021–1026). Fractions containing the polymerase were identified by gel electrophoresis of samples followed by silver-staining. FIG. 1 shows the polymerase preparation after CsCl centrifugation. Bovine serum albumin (BSA) was added during dialysis to protect against protein loss. Densitometric scanning of lane 4 compared to known quantities of whole virus in lanes 1 and 2 allowed us to estimate that the proteins in lane 4 consist of 150 ng of NP and about 1 ng total of the three polymerase proteins. One fifth of the preparation used for this gel was used per reaction.

The overall design of the plasmids used to prepare template RNAs in this study is depicted in FIG. 2. The entire insert was prepared using oligonucleotides from a DNA

synthesizer which were then cloned into the polylinker of pUC19. The insert contained a truncated promoter sequence recognized by the bacteriophage T7 RNA polymerase (Studier and Dunn, 1983, Cold Spring Harbor Symposia on Quantitative Biology, XLVII, 999–1007) so that the first nucleotides synthesized were the terminal 22 nucleotides (nt) of the conserved sequence from the 5' end of the genome RNA. When the plasmid was cut with restriction endonuclease MboII (which cuts 7 bases upstream of its recognition site), the RNA which resulted from T7 RNA polymerase transcription ended with the terminal 3' nucleotides of the influenza viral sequence. Included in the sequence was the poly-U stretch adjacent to the 5'end of the conserved terminus which is thought to comprise at least part of the termination-polyadenylation signal (Robertson, et al., 1981, J. Virol. 38, 157–163). The total length of this model genomic RNA was 53 nt since a 16 nt spacer separated the terminal conserved sequences. The model RNA which contained both termini identical to those of vRNA was named V-wt. The RNA M-wt encoded the exact complementary strand of V-wt so that the termini match those of complementary RNA (cRNA). V-wt and M-wt were constructed to serve as models for influenza virus-specific vRNA and cRNA, respectively.

6.2.2. Viral Polymerase Catalyzes Synthesis of a Full Length Copy of the Template

In the reaction using the influenza viral polymerase, V-wt template and ApG primer, a product was obtained which comigrated with a 53 nt RNA on denaturing gels. RNA migrating as a doublet at a position of about 40 to 45 nucleotides (FIG. 3A, lane 2) was also seen. This shorter product is shown below to be RNA which had terminated at a stretch of adenosines present between nucleotides 43–48 in the virion sense template. In addition to the template specific transcripts, a general background of light bands could be seen which correspond to truncated RNA products transcribed from viral genomic RNA not removed during the CsCl-glycerol centrifugation step. When no primer is used, there was no specific transcription product seen (FIG. 3A, lane 3). Additional experiments showed globin mRNA, containing a terminal cap 1 structure, was inactive as primer using initial preparations of polymerase.

When the polymerase reaction was terminated by the addition of excess buffer favorable for nuclease S1 digestion and nuclease was added, the radioactively-labeled product was resistent to digestion (FIG. 3B, lane 2). By contrast these conditions very efficiently digested the V-wt single-stranded RNA radioactively synthesized with T7 RNA polymerase (FIG. 3B, lanes 3 and 4). These nuclease S1 data confirmed that the opposite strand was indeed being synthesized in these reactions. The product of the reaction might be a double stranded RNA, but it could not be ruled out that the product was in fact single stranded and later annealed to the template RNA in the presence of high salt used in the nuclease reaction.

The RNA products were purified by electrophoresis on an 8% gel, excised, eluted from the gel, and then digested by ribonuclease T1. Products were analyzed by electrophoresis and compared to the patterns generated by RNase T1 digestion of internally labeled M-wt and V-wt control probes. As can be seen in FIG. 3C, the full length RNA (lane 1) has the identical pattern as does the plus sense RNA, M-wt (lane 3), and it does not have the pattern of the V-wt RNA (lane 4). The observed patterns were essentially identical to that which is predicted from the sequence of the RNA and thus showed that the polymerase faithfully copied the V-wt template. The smaller RNA product, a doublet with most templates, was also digested with RNase T1. Its pattern was similar to that of the full length RNA product (FIG. 3C, lane 2) except the 14 base oligonucleotide was not present. Instead, a faint 13 base oligonucleotide was seen, thus mapping the termination of the short RNA to position 44, a site where two uridines would be incorporated. Since the amount of smaller RNA product decreased at higher UTP concentrations and disappeared when CTP was used as label, these bands appeared to be an artifact of low UTP concentrations in the polymerase reaction.

6.2.3. Conditions for the Polymerase Reactions Using Model RNA Templates

It was found that protein samples containing about 30 ng of NP protein and about 200 pg total of the three P proteins would react optimally with 5 to 10 ng of RNA. By using cold competitor RNA, polyI-polyC, it was found that excess RNA nonspecifically inhibited transcription, possibly via non-specific binding of the NP protein (Kingsbury, et al., 1987, Virology 156: 396–403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11–16). In the absence of nonspecific competitor, variations in the amount of template between 1 and 10 ng produced little change in the efficiency of RNA synthesis. The NP protein and RNA were present at about equal molar concentrations and these were each about a thousand-fold in excess of the moles of the complex (assuming it to be 1:1:1) formed by the three P proteins in the typical reaction.

Since these reconstituted RNPs were able to use ApG but not globin mRNA as primer, we tested these model RNPs for other variables of the transcription reaction. In all other ways tested, the reconstituted RNPs behaved in solution similarly to those RNPs purified from detergent disrupted virus. The optimum temperature for RNA synthesis was 30° C. (FIG. 4A, lane 2) as has been repeatedly found for the viral polymerase (Bishop, et al., 1971, J. Virol. 8: 66–73; Takeuchi, et al., 1987, J. Biochem 101: 837–845; Ulmanen, et al., 1983, J. Virol. 45: 27–35). Also, the most active salt conditions were 60 mM NaCl (FIG. 4B, lane 2), again consistent with conditions used by several groups (Bishop, et al., 1971, J. Virol. 8: 66–73; Honda, et al., 1988, J. Biochem. 104: 1021–1026; Shapiro, and Krug, 1988, J. Virol. 62: 2285–2290). FIG. 4C shows a time-course experiment. The amount of RNA synthesis appeared to increase roughly linearly for the first 90 minutes, as was found for viral RNPs (Takeguchi, et al., 1987, J. Biochem. 101: 837–845).

6.2.4. Specificity of the Elongation Reaction

Various RNAs were tested for suitability as templates for the RNA polymerase of influenza virus. The pV-wt plasmid clone was digested with either EcoRI, PstI or SmaI, and T7 polymerase was used to transcribe RNA. This resulted in RNAs identical to V-wt except for the addition of 5, 13 and 38 nt at the 3' end. In FIG. 5A an overexposure of an autoradiograph is shown in order to demonstrate that no transcripts over background were observed in reactions which contained as template: two of the RNAs identical to V-wt except they contained 13 and 38 nt of extra sequence on the 3' terminus (lanes 1 and 2); a single stranded DNA of identical sequence to that of V-wt (lane 4); and an unrelated 80 nt RNA generated by transcribing the polylinker of pIBI-31 with T3 RNA polymerase (lane 5). However, the V-Eco template, containing five extra nucleotides on the 3' end, could be recognized and faithfully transcribed, although at approximately one-third the efficiency of the wild type V-wt RNA (FIG. 5B, lane 3). It is interesting to note that initiation on the V-Eco RNA by the influenza viral poly-

merase appeared to occur at the correct base since the transcribed RNA was the same size as the product from the V-wt template.

6.2.5. Analysis of the Promoter Region for the Viral RNA Polymerase

The original construct used for these studies contained the sequences of both RNA termini of genomic RNAs which could base pair and thus form a panhandle. This was done since it was shown that the vRNA in virions and in RNPs in infected cells was in circular conformation via the 15 to 16 nt long panhandle (Honda, et al., 1988, J. Biochem. 104: 1021–1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140–8144). It was further shown that the viral polymerase was bound to the double stranded structure (Honda, et al., 1988, J. Biochem. 104: 1021–1026), thus leading to the suggestion that the promoter for RNA synthesis was the panhandle. In order to test whether the panhandle was an absolute requirement for recognition, the following templates were used: the plasmid pV-wt was digested with DraI prior to transcription by the T7 polymerase (FIG. 2). This should result in an RNA molecule of 32 nt containing only virus-specific sequences from the 5' end of the RNA. When this RNA was used as template, no apparent product was produced (FIG. 5B, lane 2). Therefore the 3' terminus of virion RNA was required for this reaction. This finding was consistent with the fact that the initiation site at the 3' end of V-wt was not present in V-Dra. A second plasmid clone was produced which deleted the 5' terminal sequences but kept intact the 3' terminus. This clone, pV-d5', when digested with MboII and used for transcription by T7 polymerase produced a major transcript of 30 nt and minor species of 29 and 31 nt. Surprisingly, this template was recognized and copied by the influenza viral polymerase. FIG. 7, lane 1, shows that the product of the viral RNA polymerase reaction with V-d5' contains multiple bands reflecting the input RNA. When the products shown in FIG. 7, lane 1, were eluted from gels and subjected to RNase T1 analysis, the pattern expected of the transcription product of V-d5' was observed. Since the V-d5' RNA template was copied, the panhandle was not required for viral polymerase binding and synthesis.

Although the 5' terminus was not required for synthesis by the polymerase, a distinct possibility was that V-wt RNA might be a preferred template as compared to V-d5'. In order to examine this, reactions were done in which the templates were mixed. The V-wt RNA was present at 5 ng in each reaction. The V-d5' was absent (FIG. 6, lane 1) or was present at a 1/5 molar ratio (FIG. 6, lane 2) or a 1/1 molar ratio (FIG. 6, lane 3). The relative intensities of the bands from each RNA were determined by densitometry of the autoradiograph. The values were corrected for the amount of the radioactive nucleotide, UTP, which could be incorporated into each product, and the value was normalized so that the level of synthesis in each lane was set equal to one. The level of copying of V-wt decreased as V-d5' was increased. When V-d5' was present in one fifth molar ratio, its corrected level of synthesis was about one fourth of that from V-wt (FIG. 6, lane 2). When the two templates were present in equimolar amounts, the level of synthesis from V-wt was about 60% of the total (FIG. 6, lane 3) which might be within the expected range of experimental error for equivalent levels of synthesis. Similar results were obtained when V-d5' RNA was kept constant and the V-wt RNA was varied. It was thus concluded that the panhandle-containing V-wt RNA was not greatly favored over the template RNA which only contained the proper 3' terminus.

6.2.6. The Viral Polymerase Does Not Copy RNA Templates Containing Plus-Sense Termini

As described earlier, the influenza RNA polymerase performs three distinct activities during the course of an infection. Two activities involve the transcription of genome sense RNA and the third involves copying of the complementary sense RNA into vRNA. We therefore constructed an RNA template which contained the 5' and 3' termini of the complementary sense RNA of segment 8 (M-wt; FIG. 2).

When the M-wt RNA was used as template, little synthesis was observed (FIG. 5B, lane 4). In two experiments used for quantitation, the average level of synthesis from M-wt RNA was 4% that of V-wt. In comparing the V-wt and M-wt RNA promoters, the M-wt has only three transition changes and one point insertion within the 3' 15 nucleotides. These include a G to A change at position 3, a U to C change at position 5, a C to U change at position 8 and an inserted U between the ninth and tenth nucleotides (see Table II, below). In order to determine which of the four point differences in the 3' termini were responsible for the specificity, many combinations of these were prepared and assayed for efficiency as a template (FIG. 7). These templates were derivatives of V-d5' since they did not contain the 5' terminus. The results of densitometry scans of several experiments are outlined in Table II.

TABLE II

QUANTITATIVE COMPARISON OF THE EFFECT OF POINT MUTATIONS IN THE PROMOTER SEQUENCE*

Template	3' sequence	Level of RNA Synthesis
V-d5'	CACCCUGCUUUUGCU-OH	1
V-A3	CACCCUGCUUUUACU-OH	0.4
V-C5	CACCCUGCUUCUGCU-OH	1.0
V-d$U_{25}U_8$	CACCCUGUUUUUGCU-OH	1.0
V-U_8A_3	CACCCUGUUUUUACU-OH	0.08
V-U_8C_5	CACCCUGUUUCUGCU-OH	0.3
V-iU_{10}	CACCCUUGCUUUUGCU-OH	0.7
V-i$U_{10}A_3$	CACCCUUGCUUUUACU-OH	0.06
V-i$U_{10}U_8A_3$	CACCCUUGUUUUUACU-OH	0.2
V-i$U_{10}U_8C_5A_3$	CACCCUUGUUUCUACU-OH	0.2

*Sequences of V-wt, M-wt and V-d5' are shown in FIG. 2. All other RNAs are identical to V-d5' except for the indicated positions. The subscripted number indicates the distance from the 3' end of a change, and d and i refer to deleted or inserted nucleotides.

As shown in Table II, single point changes in V-d5' were equally well copied as compared to V-d5' itself, except for the V-A_3 RNA which was copied at 40% efficiency (FIG. 7, lane 10; Table II). When RNAs with two changes were tested, the activity generally dropped to very low levels (FIG. 7, lanes 3, 4, and 5). Therefore, these experiments confirmed that the specificity of the reactions for V-wt over M-wt was the result of the combination of the nucleotide changes present at the 3' terminus of M-wt.

6.2.7. Cap-Endonuclease Primed RNA Synthesis

The method of purifying the viral polymerase was modified in order to decrease loss of protein during dialysis. Rather than using the Amicon centricon-10 dialysis system, the enzyme was dialyzed in standard membranes resulting in higher concentrations of all four viral core proteins. The pattern of the protein gel of this preparation was identical to that shown in FIG. 1, lane 4, except that there is no BSA-derived band. It was found that 5 μl of this preparation, containing 150 ng of NP and 5 ng total of the three polymerase proteins, reacted optimally with 10 to 40 ng of model RNA template. However, the use of higher levels of protein increased the background, possibly due to higher levels of contaminating RNAs (virion RNAs not removed by CsCl centrifugation) yielding products of the size class

around 50–75 nt, complicating analysis of RNA templates containing a length of 50 nt.

This high concentration polymerase preparation was now active in cap-endonuclease primed RNA synthesis (FIG. 8A, lane 4) and also in primer-independent replication of the template RNA (FIG. 8A, lane 2). When globin mRNA was used as primer for transcription from the 30 nt V-d5' template, a triplet of bands of size about 42 to 44 nt was apparent as product (FIG. 8A, lane 4), consistent with cleavage of the cap structure at about 12 nt from the 5' end of the mRNA and use of this oligonucleotide to initiate synthesis from the 30 nt model template. Since excess RNA inhibits RNA synthesis, probably via nonspecific binding of NP in vitro as discussed above, the optimal amount of cap donor RNA added to each reaction was found to be 100 ng, which is much lower than is usually used with preformed RNP structures (e.g., Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). The most effective primer was ApG (FIG. 8A, lane 5 and lighter exposure in lane 6). The product migrates slower than that of the input template (FIG. 8A, lane 1) or the product in the absence of primer (FIG. 8A, lane 2) probably since the 5' terminus of the ApG product is unphosphorylated. The intensity of the ApG-primed product was about ten-fold higher than that of the cap-primed product, but at 0.4 mM, ApG was at a 60,000-fold molar excess of the concentration of the cap donors. Thus, although the intensity of the product band from cap-priming was about ten-fold lower than that from ApG priming, the cap-primed reaction was about 6000-fold more efficient on a molar basis. This value is similar to the approximately 4000-fold excess efficiency observed previously for the viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). It has been previously shown that cap donor RNAs containing a cap 0 structure, as in BMV RNA, are about ten-fold less active in priming the influenza viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). This unusual cap specificity was shared by the reconstituted RNPs studied here as the specific product from the model RNA was greatly decreased in reactions containing BMV RNA as cap donor. A 30 nt product was observed in lanes 2–4, probably due to primerless replication of the model template.

That the product RNAs were of the opposite sense of the input template V-ds' was shown by nuclease S1 analysis (FIG. 8B). The ApG-primed (FIG. 8B, lanes 1 and 2) and the primerless (FIG. 8B, lanes 3 and 4) RNA products were essentially nuclease resistent. The product of the cap-primed reaction (FIG. 8B, lanes 5 and 6) was partially sensitive to nuclease as about 12 nt were digested from the product. These results were most consistent with the 5' 12 nt being of mRNA origin as has been shown many times for influenza virus- specific mRNA synthesis.

The promoter specificity of this polymerase preparation in reactions primed with ApG was found to be essentially identical to those for the lower concentration enzyme as shown earlier. However, attempts thus far to perform similar analyses of promoter specificity with the primerless and cap-primed reactions have been frustrated by the comparatively high levels of background, thus making quantitation difficult.

6.2.8. Replication of Genomic Length RNA Templates

A full-length 890 nt RNA identical to the sequence of A/WSN/33 segment 8 was prepared by T7 RNA polynierase transcription of plasmid DNA, pHgaNS, which had been digested with restriction endonuclease HgaI. This RNA was copied in ApG-primed reactions containing 10 μl of the high concentration polymerase (FIG. 9, lane 8). That the RNA was in fact a copy of the template was demonstrated by its resistance to nuclease SI (FIG. 9, lane 9). A similar product was observed in the absence of primer (FIG. 9, lanes 2 and 3). Confirmation that these product RNAs were full length copies of the template was done by RNase T1 analysis. Virion RNA purified from phenol-extracted A/PR/8/34 virus was similarly copied in ApG primed reaction (FIG. 9, lanes 10 and 11) and in the absence of primer (FIG. 9, lanes 4 and 5). Interestingly, the product from replication of the HA gene was at greatly reduced levels. The 3' end of this RNA differs from that of segment 8 only at nucleotides 14 and 15, suggesting importance for these nucleotides in the promoter for RNA synthesis. In addition, we found that when whole viral RNA was used in the reconstituted RNPs, the level of acid precipitable counts was about 70% of that observed with native RNPs. The viral polymerase was also able to copy these full length RNAs when globin mRNA was used in cap-primed reaction.

7. EXAMPLE: EXPRESSION AND PACKAGING OF A FOREIGN GENE BY RECOMBINANT INFLUENZA VIRUS

The expression of the chloramphenicol transferase gene (CAT) using rRNPs is described. The rRNPs were prepared using pIVACAT (originally referred to as pCATcNS), a recombinant plasmid containing the CAT gene. The pIVACAT plasmid is a pUC19 plasmid containing in sequence: the T7-promoter; the 5'-(viral-sense) noncoding flanking sequence of the influenza A/PR8/34 RNA segment 8 (encodes the NS proteins); a BglI cloning site; the complete coding sequence of the chloramphenicol transferase (CAT) gene in the reversed and complemented order; the 3'-(viral-sense) noncoding NS RNA sequence;

and several restriction sites allowing run-off transcription of the template. The pIVACAT can be transcribed using T7 polymerase to create an RNA with influenza A viral-sense flanking sequences around a CAT gene in reversed orientation.

The in vivo experiments described in the subsections below utilized the recombinant RNA molecule described containing sequences corresponding to the untranslated 3' and 5' terminal sequences of the NS RNA of influenza virus A/PR/8/34 flanking the antisense-oriented open reading frame of the CAT gene. This RNA was mixed with purified influenza virus polymerase complex and transfected into MDCK (or 293) cells. Following infection with influenza A/WSN/33 virus, CAT activity was measured in the RNP-transfected cells and amplification of the gene was indicated. In addition, the recombinant influenza virus gene was packaged into virus particles, since CAT activity was demonstrated in cells following infection with the recombinant virus preparation.

7.1. Materials and Methods

In order to get the flanking sequences of the NS RNA fused to the coding sequence of the CAT gene, the following strategy was used. Two suitable internal restriction sites were selected, close to the start and stop codon of the CAT gene, that would allow the replacement of the sequences flanking the CAT gene in the pCM7 plasmid with the 3'- and 5'- NS RNA sequences. At the 5' end, a SfaNI site was chosen (which generates a cut 57 nt from the ATG), and at the 3'- end a ScaI site which generates a cut 28 nt from the end of the gene (stop codon included). Next, four synthetic oligonucleotides were made using an Applied Biosystems

DNA synthesizer, to generate two double-stranded DNA fragments with correct overhangs for cloning. Around the start codon these oligonucleotides formed a piece of DNA containing a XbaI overhand followed by a HaI site and a PstI site, the 3'- (viral-sense) NS sequence immediately followed by the CAT sequence from start codon up to the SfaNI overhang (underscored). In addition a silent mutation was incorporated to generate an Acci site closer to the start codon to permit future modifications.

```
Xba I
     Hga I    Pst I                               Acc
     5'-ctagacgccctgcagcaaaagcagggtgacaaagacataatggagaaaaaaatcac
     3'tgcgggacgtcgttttcgtcccactgtttctgtattacctctttttttagtg
I             SfaN I
tgggtataccaccgttgatatatcccaatcgcatcgtaaa-3'                      oligo2
acccatatggtggcaactatatagggttagcgtagcatttcttg-5'                  oligo1
```

Around the stop codon the two other oligonucleotides generated a piece of DNA as follows: a blunt-ended SCAI site, the CAT sequence from this site up to and including the stop codon (underlined) followed by a BglII site and a Xba I overhang.

```
     Sca I                          Bgl II
     5'-actgcgatgagtggcagggcggggcgtaatagat-3'           oligo3
     3'-tgacgctactcaccgtcccgccccgcattatctagatc-5'       oligo4
                                      Xba I
```

Using a single internal EcoRI site in the CAT sequence, the SfaNI/EcoRI and the EcoRI/ScaI fragment from pCM7 were independently cut out and purified from acrylamide gels. The SfaNI/EcoRI fragment was subsequently ligated with the synthetic DNA fragment obtained by annealing oligonucleotides 1 and 2 into a pUC19 plasmid that was cut with XbaI and EcoRI. The EcoRI/ScaI fragment was similarly cloned into an XbaI and EcoRI-digested pUC19 plasmid using oligonucleotides 3 and 4. The ligated DNA was transformed into competent DH5a bacteria, amplified, isolated and screened by means of restriction analysis using standard techniques.

The recombinants with the SfaNI containing insert were cut with XbaI and EcoRI and the plasmids with the ScaI insert were cut with EcoRI and BglII. The fragments were purified from acrylamide gel and cloned together into the pPHV vector which had been cut with XbaI and BglII. After transformation, white colonies were grown, analyzed by endonuclease digestion and selected clones were sequenced. The final clone, pCATcNS2, was grown in large amounts and sequenced from the flanking pUC sequences up to 300 nt into the CAT gene, revealing no discrepancies with the intended sequence, with the exception of a G to A transition in the CAT gene, which appeared silent.

7.1.1. Viruses and Cells

Influenza A/PR/8/34 and A/WSN/33 viruses were grown in embryonated eggs and MDCK cells, respectively (Ritchey et al. 1976, J. Virol. 18: 736–744; Sugiura et al., 1972, J. Virol. 10: 639–647). RNP-transfections were performed on human 293 cells (Graham et al., 1977, J. Gen. Virol. 36: 59–72) and on Madin-Darby canine kidney (MDCK) cells (Sugiura et al., 1972, supra).

7.1.2. Construction of Plasmids

Plasmid pIVACAT1, derived from pUC19, contains the coding region of the chloramphenicol acetyltransferase (CAT) gene flanked by the noncoding sequences of the influenza A/PR/8/34 RNA segment 8. This construct is placed under the control of the T7 polymerase promoter in such a way that the RNA transcript IVACAT1 contains in 5to 3' order: 22 nucleotides derived from the 5' terminus of the influenza virus NS RNA, an 8 nt linker sequence including a BglII restriction site, the CAT gene in negative polarity, and 26 nt derived form the 3' end of the influenza virus NS RNA (FIG. 11).

pIVACAT1 was constructed in the following way: In order to obtain the correct 5'-end in pIVACAT1, the EcoRI-ScaI fragment of the CAT gene derived from plasmid pCM7 (Pharmacia) was ligated to a DNA fragment formed by two synthetic oligonucleotides. The sequence of these oligonucleotides are:

5'- ACTGCGATGAGTGGCAGGGCGGGGCGTAATA—GAT-3'

(top strand), and

5'- CTAGATCTATTACGCCCCGCCCTGCCAC—TCATCGCAGT-3'

(bottom strand). For the 3'-end of the insert in pIVACAT1 the SfaN 1-EcoRI fragment of the CAT gene was ligated to a DNA fragment made up of the synthetic oligonucleotides:

5'- CTAGACGCCCTGCAGAAAAGCAGGGTGAC — AAAGACA TAATGGAGAAAAAAAATCACTGGGTATACCACCGTTGATAT ATCCCAATCG — CATCGTAAA-3'

(top strand), and

5'- GTTCTTTACGATGCGATTGGGAT—TTTGCTGCAGGGCGT-3'

(bottom strand). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. These 5' and 3' constructs were ligated into pUC19 shuttle vectors digested with XbaI and EcoRI, grown up, cut out with EcoRI/BIII (5' region) and XbaI/EcoRI (3' region) and ligated into BIII/XbaI cut pPHV. The latter plasmid is similar to pV-WT described in Section 6, supra, except that it contains a BglII site which separates the noncoding terminal sequences of the influenza A virus NS RNA segment. The final clone pIVACAT1 (FIG. 1) was grown up and the DNA was partially sequenced starting from the flanking pUC sequences and reaching into the CAT gene. No changes were found as compared to the expected sequences with the exception of a silent G to A transition in the CAT gene at position 106 relative to the start of the IVACAT1 RNA.

7.1.3. T7 RNA Transcription

Plasmid pIVACAT1 was digested with HgaI (FIG. II), to allow run-off transcription. The 5 nt overhang generated by this enzyme was filled in with Klenow enzyme (BRL) and the DNA was purified over a spin column (Boehringer). The T7 polymerase reaction was performed using standard procedures in the presence of Rnasin (Promega). Template DNA was removed from Rnase free Dnase I (Promega). The RNA was purified over Qiagen tip-5 columns (Qiagen, Inc.) and quantitated using 4% polyacrylamide gels which were silver stained. NS RNA was prepared from plasmid pHgaNS in the same way.

7.1.4. Purification of Influenza A Virus Polymerase and In Vitro Transcription

The RNA polymerase complex was purified from influenza A/PR/8/34 as described in Section 6, supra. In vitro transcriptions of cold IVACAT1 or HgaNS RNA template were carried out using the conditions which have been described in Section 6, supra. Radiolabeled transcripts were analyzed on 4% acrylamide gels.

7.1.5. RNP-Transfection of MDCK and 293 Cells

35 mm dishes containing approximately 10^6 cells were treated with 1 ml of a solution of 300 μg/ml DEAE-dextrin,

0.5% DMSO in PBS/gelatine (0.1 mg/ml gelatine) for 30 minutes at room temperature. After removal of this solution, 200 μg of μl PBS/gelatine containing 1 μg IVACAT 1 RNA (1–2 μl), 20 μl of the purified polymerase preparation and 4 μl of Rnasin was added to the cells and incubated for 1 hour at 37° C. This was followed by the addition of gradient purified influenza A/WSN/33 virus (moi 2–10). After incubation for one hour at 37° C., 2.5 ml of either DMEM+10% FCS media (293 cells) or MEM media (MDCK cells) was added. In some experiments MDCK cells were first infected and subsequently RNP-transfected. Harvesting of cells was done in NET buffer or in media, using a rubber policemen (MDCK cells), or by gentle suspension (293 cells). Cells were spun down and the pellets were resuspended in 100 μl of 0.25 M Tris buffer, pH 7.5. The samples were subsequently freeze-thawed three-times and the cell debris was pelleted. The supernatant was used for CAT assays.

7.1.6. Passaging of Virus from RNP-Transfected Cells

MDCK cells were infected with helper virus and RNP-transfected 2 hours later as described above. After 1 hour cells and media were collected and cells were spun down. 100 μl of the supernatant media, containing virus, was added to 35 mm dishes with MDCK cells. After 12 hours these cells and media were collected and assayed for CAT activity. Virus contained in this supernatant media was used for subsequent rounds of infection of MDCK cells in 35 mm dishes.

7.1.7. CAT Assays

CAT assays were done according to standard procedures, adapted from Gorman et al., 1982, Mol. Cell. Biol. 2: 1044–1051. The assays contained 10 μl of ^{14}C chloramphenicol (0.5 μCi; 8.3 nM; NEN), 20 μl of 40 mM acetyl CoA (Boehringer) and 50 μl of cell extracts in 0.25M Tris buffer (pH 7.5). Incubation times were 16–18 hours.

7.2. Results

rRNA templates were prepared from HgaI digested, end filled linearized pCATcNS using the bacteriophage T7 RNA polymerase as described in Section 6. The rRNA templates were combined with the viral RNA polymerase complex prepared as described in Section 6.1.1., and the resulting rRNPs were used to transfect MDCK and 293 cells lines which were superinfected with influenza A/WSN33. In each cell line transfected with the rRNPs, high levels of expression of CAT was obtained 6 hours post-infection. In addition, virus stocks obtained 24 hours post-infection synthesized high levels of CAT enzyme after subsequent passage in MDCK cells. The CAT-RNP was packaged into virus particles.

7.2.1. Synthesis of IVACAT1 Template RNA

In order to study the transcription and replication signals of influenza A virus RNAs in vivo, we constructed plasmid pIVACAT1 (FIG. II) which directs the synthesis of an NS RNA-like transcript. This RNA shares the 22 5' terminal and the 26 3' terminal nucleotides with the NS RNA of influenza A/PR/8/34 virus and contain—instead of the coding sequences for the NS1 and NS2 proteins—those for a full-length CAT protein. For cloning purposes it also contains eight additional nucleotides including a BglII site between the stop codon of the CAT gene and the stretch of U's in the 5' noncoding region. The 17 promoter adjacent to the 5' noncoding sequences and the HgaI site downstream of the 3' end allow for the exact tailoring of the 5' and 3' ends. Run-off transcription using T7 polymerase generates a 716 nt long RNA: FIG. 12, lanes 2–4 show that this RNA is of discrete length and shorter than the 890 nt long marker NS RNA, which was synthesized by T7 transcription of pHgaNS (lane 1).

7.2.2. The IVACAT1 RNA is Transcribed In Vitro by the Influenza a Virus RNA Polymerase

In the examples described in Section 6, it was demonstrated that synthetic RNAs containing at the 3' end the 15 3' terminal nucleotides of influenza virus RNA segment 8 can be transcribed in vitro using purified influenza A virus RNA polymerase. We tested whether unlabeled IVACAT1 RNA could be transcribed in a similar way. FIG. 12 lane 5 shows that the in vitro transcription reaction generated an RNA of discrete length and similar size to the product of the T7 transcription reaction suggesting synthesis of a full length product.

7.2.3. RNP-Transfection and CAT Activity

Since the recombinant CAT RNA could be transcribed in vitro, a system was designed to test whether this RNA can be recognized and replicated in vivo (FIG. 13). Recombinant RNA was mixed with the purified polymerase to allow formation of viral RNP-like particles. To facilitate the association, the RNA/polymerase mixture was incubated in transcription buffer without nucleotides for 30 minutes at 30° C. prior to RNP-transfection. In some experiments, this preincubation step was omitted. RNP-transfections were either preceded or followed by infection with influenza A/WSN/33 virus, since the production of viral polymerase protein was expected to be necessary for efficient amplification of the gene. The cells used were either MDCK cells, which are readily susceptible to influenza A/WSN/33 virus infection, or human 293 cells, which support infection at a slower rate.

In order to determine whether the minus sense IVACAT1 RNA could be amplified and transcribed in vivo, an experiment was performed in 293 cells. Cells were transfected with RNP, virus infected one hour later and harvested at various times post-infection. FIG. 14A shows that at early times post infection only background levels of CAT activity were detected (lanes 5, 7 and 9). However, significant levels of CAT activity appeared seven hours after virus infection (lane 11) A similar level of CAT activity was detected two hours later (lane 13). There were background levels of CAT activity in the mock transfected 5 cells at any time point (lanes 6, 8, 10, 12 and 14), and in control cells not infected with A/WSN/33 virus (lanes 1–4).

Preincubation of RNA and polymerase complex was not necessary for successful RNP-transfection. As can be seen in FIG. 14B, lanes 2 and 3, preincubation might actually cause a decrease in CAT activity, presumably due to RNA degradation during preincubation. In another control experiment, infection by helper virus of RNP-transfected cells was omitted (FIG. 14B, lanes 4 and 5). Since these lanes show no CAT activity we conclude that the IVACAT1 RNA is amplified specifically by the protein machinery supplied by the helper virus. In an additional control experiment, naked RNA was transfected into cells which were subsequently helper-infected or mock-infected. Again, no CAT activity was detected in these samples (FIG. 14B, lanes 6–9). Finally virus-infected cells which were not transfected with recombinate CAT-RNP also did not exhibit endogenous acetylation activity (FIG. 14B, lane 10). It thus appears that addition of the purified polymerase to the recombinant RNA as well as infection of cells by helper virus is important for successful expression of the CAT enzyme.

Experiments were also performed using MDCK cells, the usual tissue culture host cell for influenza virus (FIG. 14C). When the reconstituted recombinant CAT-RNP complex was transfected 1 hour before virus infection, little CAT activity was observed at 7 hours post virus infection (FIG.

14C, lane 1). When RNP-transfection was accomplished 2 hours after virus infection, expression of CAT was greatly enhanced at 7 hours post-virus infection (FIG. **14C**, lane 3). Therefore, MDCK cells are also viable host cells for these experiments.

7.2.4. The CAT-RNP is Packaged into Virus Particles

Since the recombinant CAT RNA can be replicated in vivo via helper virus functions, we examined whether virus produced in RNP-transfected and helper virus infected cells contained the CAT gene. MDCK cells were used in the experiment because they yield higher titers of infectious virus than 293 cells. MDCK cells were infected with A/WSN/33 virus, RNP-transfected 2 hours later and allowed to incubate overnight. At 14 hours post infection, media was harvested and cells were pelleted. Virus supernatant was then used to infect new MDCK cell monolayers. The inoculum was removed after 1 hour and cells were harvested at 12 hours post infection and assayed for CAT activity. FIG. **15** reveals that the virus preparation induces a level of CAT activity (lanes 2 and 3) which is significantly above control (lane 1). In this case, the addition of helper virus to the inoculum did not increase CAT activity (lane 4). Further passaging of supernatant virus on fresh MDCK cells did not result in measurable induction of CAT activity. This is not surprising as there is no selective pressure for retaining the CAT gene in these viral preparations. We excluded the possibility that we were transferring the original RNA/polymerase complex by pretreating the inocula with RNase. This treatment destroys viral RNPs of influenza virus (Pons et al., 1969, Virology 39: 250–259; Scholtissek and Becht, 1971 J. Gen. Virol. 10: 11–16).

8. RESCUE OF INFECTIOUS INFLUENZA VIRUSES USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs

The experiments described in the subsections below demonstrate the rescue of infectious influenza viruses using RNA which is derived from specific recombinant DNAs. RNAs corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN virus) were transcribed in vitro from appropriate plasmid DNAs and—following the addition of purified influenza virus polymerase complex (as described in Section 6.1.1. supra)—were transfected into MDBK cells as described in Section 7, supra. Superinfection with helper virus, lacking the WSN NA gene, resulted in the release of viruses containing the WSN NA gene. Thus, this technology allows the engineering of infectious influenza viruses using cDNA clones and site-specific mutagenesis of their genomes. Furthermore, this technology may allow for the construction of infectious chimeric influenza viruses which can be used as efficient vectors for gene expression in tissue culture, animals or man.

The experiments described in Sections 6 and 7 supra, demonstrate that the 15 3' terminal nucleotides of negative strand influenza virus RNAs are sufficient to allow transaction in vitro using purified influenza virus polymerase proteins. In addition, the studies using the reporter gene chloramphenicol acetyltransferase (CAT) show that the 22 5' terminal and the 26 3' terminal nucleotides of the viral RNAs contain all the signals necessary for transcription, replication and packaging of influenza virus RNAs. As an extension of these results, a plasmid, pT3NAv, was constructed which contained the complete NA gene of influenza A/WSN/33 virus downstream of a truncated T3 promoter (FIG. **16**). Therefore, runoff transcription of this plasmid, cut at the Ksp632I site, yields an RNA which is identical to the true genomic NA gene of the WSN virus (FIG. **17**, lane 3). This RNA was then incubated with purified polymerase (purified as described in Section 6.1.1) and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus which did not contain the WSN virus NA. The choice of WSN-HK helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HK virus can only form plaques in MDBK cells when protease is added to the medium. This is in marked contrast to WSN virus (isogenic to WSN-HK virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Schulman et al., 1977, J. Virol. 24: 170–176).

8.1. Materials and Methods

8.1.1. Viruses and Cells

Influenza A/WSN/33 virus and A/WSN-HK virus were grown in Madin-Darby canine kidney (MDCK) cells and embryonated eggs, respectively (Sugiura et al., 1972, J. Virol. 10: 639–647; Schulman et al., 1977, J. Virol. 24: 170–176). Influenza A/PR/8/34 virus was also grown in embryonated eggs. Madin-Darby bovine kidney (MDBK) cells were used for the transfection experiments and for selection of rescued virus (Sugiura et al., 1972, J. Virol. 10: 639–647).

8.1.2. Construction of Plasmids

The pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 plasmids were constructed by PCR-directed mutagenesis using a cloned copy of the WSN NA gene, which was obtained following standard procedures (Buonagurio et al., 1986, Science 232: 980–982). To construct pT3NAv, the following primers were used:

5'-CGGAATTCTCTTCGAGCGAAAGCAGGAGTT-3' and

5'-CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTT-3'.

After 35 cycles in a thermal cycler (Coy Lab Products, Mich.), the PCR product was digested with EcoRI and HindIII and cloned into pUC19. Plasmid pT3NAv mut 1 was constructed in a similar fashion except that the sequence of the primer was altered (FIG. 16). Plasmid pT3NAv mut 2 was constructed by cassette mutagenesis through the digestion of pT3NAv with PstI and NcoI and religation in the presence of the synthetic oligonucleotides—

5'-CATGGGTGAGTTTCGACCAAAATCTAGATTATAAAATAGGATACATATGCA-3'

and 5'-AATGTATCCTATTTTATAATCTAGATTTTGGTCGAAACTCACC-3.

Oligonucleotides were synthesized on an applied Biosystems DNA synthesizer. The final clones pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 were grown up and the DNAs were partially sequenced starting from the flanking pUC 19 sequences and reaching into the coding sequences of the NA gene. The mutations in pT3NAv mut 2 were also confirmed by sequencing.

8.1.3. Purification of Influenza a Virus Polymerase and RNP Transfection in MDBK Cells

The RNA polymerase complex was purified from influenza A/PR/8/34 virus as described in Section 6. 1. 1, supra, and was then used for RNP transfection in MDBK cells using the protocol described in Section 7, supra, except that WSN-HK virus was used as helper virus at an moi of 1. RNAs used for RNP transfection were obtained by phenol extraction of purified virus or by transcription (using T3 polymerase) of pT3NAv, pT3NAv mut 1 and pT3NAv mut 2. All plasmids were digested with Ksp632I, end-filled by Klenow enzyme (BRL) and then transcribed in a runoff reaction a described in Section 7, supra.

8.2. Results

8.2.1. Rescue of Infectious Influenza Virus in MDBK Cells Using RNA Derived from Recombinant Plasmid DNA

A plasmid, pT3NAv, was constructed to contain the complete NA gene of influenza WSN virus downstream of a truncated T3 promoter (FIG. **16**). Runoff transcription of the plasmid, cut at the Ksp632I site, yields an RNA which is identical in length to the true genomic NA gene of the WSN virus (FIG. **17**, lane 3). This RNA was then incubated with purified polymerase and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus. The choice of WSN-HK virus as helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, its was shown that the WSN-HN virus can only form plaques in MDBK cells when protease is added to the medium (Schulman et al., 1977, J. Virol. 24: 170–176). This is in marked contrast to WSN virus (isogenic to WSN-HK helper virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Sugiura et al., 1972, J. Virol. 10: 639–647). MDBK cells were first infected with the WSN-HK helper virus and RNP-transfected one hour after virus infection. Following overnight incubation in the presence of 20μg/ml plasminogen, supernatant from these cells was then amplified and plaqued in MDBK cells in the absence of protease in the medium. The appearance of plaques in MDBK cells (Schulman et al., 197, J. Virol. 10: 639–647) indicated the presence of virus which contained the WSN virus NA gene, since supernatant from control experiments of cells infected only with the WSN-HK virus did not produce plaques. In a typical experiment involving the use of a 35 mm dish for the RNP-transfection, 2.5×10^2 plaques were observed.

In another control experiment, synthetic NA RNA was used which was derived from plasmid pT3NAv mut 1 (FIG. **16**). This RNA differs from the wild type NA RNA derived from pT3NAv by a single nucleotide deletion in the non-translated region of the 5' end (FIG. **16**). RNP-transfection of MDBK cells with this RNA and superinfection with WSN-HK virus did not result in the formation of rescued virus. This negative result is readily explained since we have shown in Section 6 and 7, supra, that the essential sequences for the recognition of viral RNA by viral polymerases as well as the packaging signals are located within the 3' and 5' terminal sequences of the viral RNAs. However, we cannot exclude the possibility that rescue of virus using this mutated RNA does occur, albeit at an undetected frequency.

8.2.2. RNA Analysis of Rescued Virus

Virus obtained in the rescue experiment was plaque purified, amplified in MDBK cells and RNA was extracted from this preparation. The RNA was then analyzed by electrophoresis on a polyacrylamide gel. FIG. **17** shows the RNA of the helper virus WSN-HK (lane 1) and the synthetic NA RNA (lane 3), which was transcribe by T3 polymerase from plasmid pT3NAv. The migration pattern of the RNAs of the rescued virus (lane 2) is identical to that of control WSN virus (lane 4). Also, the NA RNAs in lanes 2 and 4 migrate at the same position as the NA RNA derived from cDNA (lane 3) and faster than the HK virus NA band in the helper WSN-HK virus (lane 1). These experiments support the conclusion that as a result of the RNP-transfection, infectious virus was formed containing WSN virus NA RNA derived from cDNA.

8.2.3. Rescue of Infectious Influenza Virus Using Virion RNA

In another transfection experiment, RNA extracted from purified WSN virus was employed. When this naked RNA is transfected together with the polymerase proteins into helper virus infected cells, rescue of WSN virus capable of replicating in MDBK cells is observed. RNA isolated from an amplified plaque in this experiment is analyzed in lane 5 of FIG. **17** and shows a pattern indistinguishable from that of the control of WSN virus in lane 4.

8.2.4. Introduction of Site-Specific Mutations into the Viral Genome

The experiments described so far involved the rescue of influenza WSN virus. Since the synthetic RNA used in these experiments is identical to the authentic WSN NA gene, the unlikely possibility of contamination by wild type WSN virus could not be rigorously ruled out. Therefore, we introduced five silent point mutations in the coding region of the NA gene in plasmid pT3NAv. These mutations were introduced by cassette mutagenesis through replacement of the short NcoI/PstI fragment present in the NA gene. The five mutations in the cDNA included a C to T change at position 901 and a C to A change at position 925, creating a new XbaI site and destroying the original PstI site, respectively. In addition, the entire serine codon at position 887–889 of the cDNA clone was replaced with an alternate serine triplet (FIG. **17**). RMP-transfection of this mutagenized RNA (pT3NAv mut 2) and helper virus infection of MDBK cells again resulted in the rescue of a WSN-like virus which grew in MDBK cells in the absence of added protease. When the RNA of this virus was examined by sequence analysis, all five point mutations present in the plasmid DNA (FIG. **16**) were observed in the viral RNA (FIG. **18**). Since it is extremely unlikely that these mutations evolved in the wild type influenza WSN virus, we conclude that successful rescue of infectious influenza virus containing five site-specific mutations was achieved via RNP-transfection of engineered RNA.

9. RESCUE OF INFECTIOUS RESPIRATORY SYNCYTIAL VIRUSES (RSV) USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs.

This example describes a process for the rescue of synthetic RNAs of infectious respiratory syncytial virus (RSV), derived from recombinant cDNAs of first portions of and then the entire RSV RNA genome into stable and infectious RSVs, as noted in Section 5 above. This process can be used in the production of chimeric RSV viruses which can express foreign genes, ie, genes non-native to RSV. Another exemplary way to achieve the production of chimeric RSV involves modifying existing, native RSV genes, as is further described. Accordingly, this example also describes the utility of this process in the directed attenuation of RSV pathogenicity, resulting in production of a vaccine with defined, engineered biological properties for use in humans.

The first step of the rescue process involving portions of the RSV genome requires synthesis of double strand DNA representing the 3' and 5'-terminal extracistronic regions of RSV strain A2 (leader and trailer respectively). The assembly of these extracistronic regions is described in more detail in Section 9.1.2. The bacterial CAT gene, the exemplary heterologous gene coding sequence used in this example, is then flanked with the leader and trailer DNAs to form a miniature RSV 'genome' with the leader region positioned closest to the CAT gene AUG (initiation codon for protein synthesis); see FIG. 21. RNA is transcribed in vitro from this DNA construct and transfected into helper virus-infected (RSV strain A2, or RSV strain B9320) 293 cells. The subsequent presence of CAT activity in these cells indicates the encapsidation, transcription and replication of the RSV/CAT RNA. It will be apparent to one skilled in the art that other heterologous sequences, such as for example, other foreign or non-native genes (i.e. genes from organisms or sources other than RSV), additional RSV genes, native RSV genes or regulatory sequences having specific substitutions, additions and/or deletions in the nucleotide sequence and/or gross alterations in the genetic structure of native RSV, that, in effect, result in heterologous sequences due to their alterations or modifications, can be substituted for the CAT sequence exemplified herein to produce a chimeric RSV.

The first step of the rescue process involving the entire RSV RNA genome requires synthesis of a full length copy of the 15 kilobase (kb) genome of RSV strain A2. This is accomplished by splicing together subgenomic double strand cDNAs (using standard procedures for genetic manipulation) ranging in size from 1 kb–3.5 kb, to form the complete genome cDNA. Determination of the nucleotide sequence of the genome cDNA allows identification of errors introduced during the assembly process; errors can be corrected by site directed mutagenesis, or by substitution of the error region with a piece of chemically synthesized double strand DNA. Following assembly, the genome cDNA is positioned adjacent to a transcriptional promoter (e.g., the T7 promoter) at one end and DNA sequence which allows transcriptional termination at the other end, e.g., a specific endonuclease or a ribozyme, to allow synthesis of a plus or minus sense RNA copy of the complete virus genome in vitro or in cultured cells.

The rescue process utilizes the interaction of full length RSV strain A2 genome RNA, which is transcribed from the constructed cDNA, with helper RSV subgroup B virus proteins inside cultured cells. This can be accomplished in a number of ways. For example, full length virus genomic RNA from RSV strain A2 can be transcribed in vitro and transfected into RSV strain B9320 infected cells, such as 293 cells using standard transfection protocols. In addition, in vitro transcribed genomic RNA from RSV strain A2 can be transfected into a cell line expressing the essential RSV strain A2 proteins (in the absence of helper virus) from stably integrated virus genes.

Alternatively, in vitro transcribed virus genome RNA (RSV strain A2) can also be transfected into cells infected with a heterologous virus (e.g. in particular vaccinia virus) expressing the essential helper RSV strain A2 proteins, specifically the N, P and L proteins. In addition the in vitro transcribed genomic RNA may be transfected into cells infected with a heterologous virus, for. example vaccinia virus, expressing T7 polymerase, which enables expression of helper proteins from transfected plasmid DNAs containing the helper N, P and L genes.

As an alternative to transfection of in vitro transcribed genomic RNA, plasmid DNA containing the entire RSV cDNA construct may be transfected into cells infected with a heterologous virus, for example vaccinia virus, expressing the essential helper RSV strain A2 proteins and T7 polymerase, thereby enabling transcription of the entire RSV genomic RNA from the plasmid DNA containing the RSV cDNA construct. The vaccinia virus need not however, supply the helper proteins themselves but only the T7 polymerase; then helper proteins may be expressed from transfected plasmids containing the RSV N, P and L genes, appropriately positioned adjacent to their own T7 promoters.

Where replicating virus is providing the helper function during rescue experiments, the B9320 strain of RSV is used, allowing differentiation of progeny rescued RSV strain A2 and helper virus RSV strain B9320 with neutralizing monoclonal antibodies directed against RSV B9320. Rescued RSV strain A2 is positively identified by the presence of specific nucleotide 'marker' sequences inserted in the cDNA copy of the RSV genome prior to rescue.

The establishment of a rescue system for native, ie, 'wild-type' RSV strain A2 allows modifications to be introduced into the cDNA copy of the RSV genome to construct chimeric RSV containing sequences heterologous in some manner to that of native RSV, such that the resulting rescued virus may be attenuated in pathogenicity to provide a safe and efficacious human vaccine as discussed in Section 5.4 above. The genetic alterations required to cause virus attenuation may be gross (e.g. translocation of whole genes and/or regulatory sequences within the virus genome), or minor (e.g. single or multiple nucleotide substitution(s), addition(s) and/or deletion(s) in key regulatory or functional domains within the virus genome), as further described in detail.

In addition to alteration(s) (including alteration resulting from translocation) of the RSV genetic material to provide heterologous sequence, this process permits the insertion of 'foreign' genes (i.e., genes non-native to RSV) or genetic components thereof exhibiting biological function or antigenicity in such a way as to give expression of these genetic elements; in this way the modified, chimeric RSV can act as an expression system for other heterologous proteins or genetic elements, such as ribozymes, anti-sense RNA, specific oligoribonucleotides, with prophylactic or therapeutic potential.

9.1 Rescue of the Leader and Trailer Sequences of RSV Strain A2 Using RSV Strain B9320 as Helper

9.1.1. Viruses and Cells

Although RSV strain A2 and RSV strain B9320 was used in this Example, they are exemplary. It is within the skill in the art to use other strains of RSV subgroup A and RSV subgroup B viruses in accordance with the teachings of this Example. Methods which employ such other strains are encompassed by the invention.

RSV strain A2 and RSV strain B9320 were grown in Hep-2 cells and Vero cells respectively, and 293 cells were used as host during transfection/rescue experiments. All three cell lines were obtained from the ATCC (Rockville, Md.). The RSV strain A2 was also obtained from the ATCC and RSV strain B9320 was a gift from Dr. M. Hendry at the Department of Health Services, Berkeley, Calif.

9.1.2. Construction of Plasmids

Plasmid pRSVA2CAT (FIG. 21) was constructed as described below.

The cDNAs of the 44 nucleotide leader and 155 nucleotide trailer components of RSV strain A2 (see Mink et al., *Virology* 185:615–624 (1991); Collins et al., *Proc. Natl.*

Acad. Sci. 88:9663–9667 (1991)), the trailer component also including the promoter consensus sequence of bacteriophage T7 polymerase, were separately assembled by controlled annealing of oligonucleotides with partial overlapping complementarity (see FIG. **21**). The oligonucleotides used in the annealing were synthesized on an Applied Biosystems DNA synthesizer (Foster City, Calif.). The separate oligonucleotides and their relative positions in the leader and trailer sequences are indicated in FIG. **21**. The oligonucleotides used to construct the leader were:

1. 5' CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA AAC TTG CAT AAA C
2. 5' CAA AAA AAT GGG GCA AAT AAG AAT TTG ATA AGT ACC ACT TAA ATT TAA CT
3. 5' CTA GAG TTA AAT TTA AGT GGT ACT
4. 5' TAT CAA ATT CTT ATT TGC CCC ATT TTT TTG GTT TAT GCA AGT TTG TTG TA
5. 5' CGC ATT TTT TCG CGT AAT ATG CGT CGG TAC

The oligonucleotides used to construct the trailer were:

1. 5' GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA TAA TTT TTT AAA TA
2. 5' ACT TTT AGT GAA CTA ATC CTA AAG TTA TCA TTT TAA TCT TGG AGG AAT AA
3. 5' ATT TAA ACC CTA ATC TAA TTG GTT TAT ATG TGT ATT AAC TAA ATT ACG AG
4. 5' ATA TTA GTT TTT GAC ACT TTT TTT CTC GTT ATA GTG AGT CGT ATT A
5. 5' AGC TTA ATA CGA CTC ACT ATA ACG A
6. 5' GAA AAA AAG TGT CAA AAA CTA ATA TCT CGT AAT TTA GTT AAT ACA CAT AT
7. 5' AAA CCA ATT AGA TTA GGG TTT AAA TTT ATT CCT CCA AGA TTA AAA TGA TA
8. 5' ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT AAA AAA TTA TAT GAT TTT TA
9. 5' ATT TTT AAT AAC TAT AAT TGA ATA CTG CA

The complete leader and trailer cDNAs were then ligated to the CAT reporter gene XbaI and PstI sites respectively to form a linear~1 kb RSV/CAT cDNA construct. This cDNA construct was then ligated into the KpnI and HindIII sites of pUC19. The integrity of the final pRSVA2CAT construct was checked by gel analysis of the size of XbaI/PstI and KpnI/HindIII digestion products.

In vitro transcription of HgaI linearlized pRSVA2CAT with bacteriophage T7 polymerase was performed according to the T7 supplier protocol (Promega Corporation, Madison, Wis.). Confluent 293 cells in six-well dishes (~1×10^6 cells per well) were infected with RSV strain B9320 at 1 pfu $cell^{-1}$ and 1 hour later were transfected with 5–10 μg of the in vitro transcribed RNA from the pRSVA2CAT construct. The transfection procedure followed the transfection procedure of Collins et al., *Virology* 195:252–256 (1993) and employed Transfect/ACE™ and Optimem reagents according to the manufacturers specifications (Gibco-BRL, Bethesda, Md.). At 24 hours post infection the 293 cells were assayed for CAT activity using a standard protocol (Current Protocols in Molecular Biology, Vol. 1, Chapter 9.6.2; Gorman, et al., (1982) Mol. Cell. Biol. 2:1044–1051). The detection of high levels of CAT activity indicated that in vitro transcribed negative sense RNA containing the 'leader' and 'trailer' regions of the RSV A2 strain genome and the CAT gene can be encapsidated, replicated and expressed using proteins supplied by RSV strain B9320 (See FIG. **22**). The level of CAT activity observed in these experiments was at least as high as that observed in similar rescue experiments where homologous RSV strain A2 was used as helper virus. This ability of an antigenically distinct subgroup B RSV strain B9320 to support the encapsidation, replication and transcription of a subgroup A RSV strain A2 RNA has to our knowledge hitherto not been formally reported. In view of the fact that influenza B virus is unable to provide analogous helper activity for similar CAT constructs containing influenza A leader and trailer sequences, these observations are therefore surprising and unexpected.

9.2 Construction and Rescue of a cDNA Representing the Complete Genome of RSV

To obtain a template for cDNA synthesis, RSV genomic RNA, comprising 15,222 nucleotides, is purified from infected Hep-2 cells according to the method described by Ward et al., *J. Gen. Virol.* 64:1867–1876 (1983). Based on the published nucleotide sequence of RSV, oligonucleotides are synthesized using an Applied Biosystems DNA synthesizer (Applied Biosystems, Foster City, Calif.) to act as primers for first and second strand cDNA synthesis from the genomic RNA template. The nucleotide sequences and the relative positions of the cDNA primers and key endonuclease sites within the RSV genome are indicated in FIG. **23**. The production of cDNAs from virus genomic RNA is carried out according to the reverse transcription/polymerase chain reaction (RT/PCR) protocol of Perkin Elmer Corporation, Norwalk, Conn. (see also Wang et al., (1989) *Proc. Natl. Acad. Sci.* 86:9717–9721); the amplified cDNAs are purified by electro elution of the appropriate DNA band from agarose gels. Purified DNA is ligated directly into the pCRII plasmid vector (Invitrogen Corp. San Diego), and transformed into either 'One Shot' *E. coli* cells (Invitrogen) or 'SURE' *E. coli* cells (Stratagene, San Diego). The resulting, cloned, virus specific, cDNAs are assembled by standard cloning techniques (Sambrook et al., *Molecular Cloning—A Laboratory manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y., 1989) to produce a cDNA spanning the complete RSV genome. The entire cDNA genome is sequenced, and incorrect sequence replaced by either site-directed mutagenesis or chemically synthesized DNA. Nucleotide substitutions are introduced at bases 7291 and 7294 (with base number 1 being at the start of the genomic RNA 3' end) in the 'F' gene, to produce a novel StuI endonuclease site, and at positions 7423, 7424, and 7425 (also in the F gene) to produce a novel PmeI site. These changes are designed to act as definitive markers for rescue events. The bacteriophage T7 polymerase and the HgaI endonuclease site are placed at opposite ends of the virus genome cDNA such that either negative or positive sense virus genome RNA can be synthesized in vitro. The cDNAs representing the T7 polymerase promoter sequence and the recognition sequence for HgaI were synthesized on an Applied Biosystems DNA synthesizer and are separately ligated to the ends of the virus genome cDNA, or are added

as an integral part of PCR primers during amplification of the terminal portion of the genome cDNA, where appropriate; the latter procedure is used when suitable endonuclease sites near the genome cDNA termini are absent, preventing direct ligation of chemically synthesized T7 promoter/HgaI site cDNA to the genome cDNA. This complete construct (genome cDNA and flanking T7 promoter/HgaI recognition sequence) is then cloned into the KpnI/NotI sites of the Bluescript II SK phagemid (Stratagene, San Diego) from which the endogenous T7 promoter has been removed by site-directed mutagenesis. RNA transcribed from this complete genome construct may be rescued using RSV subgroup B helper virus to give infectious RSV in accordance with Example 9.1. This basic rescue system for the complete native, i.e., 'wild-type' RSV A2 strain genomic RNA can be employed to introduce a variety of modifications into the cDNA copy of the genome resulting in the introduction of heterologous sequences into the genome. Such changes can be designed to reduce viral pathogenicity without restricting virus replication to a point where rescue becomes impossible or where virus gene expression is insufficient to stimulate adequate immunity.

These modifications can comprise gross alterations of the genetic structure of RSV, such as gene shuffling. For example, the RSV 'M_1' gene can be translocated to a position closer to the 5' end of the genome, in order to take advantage of the known 3' to 5' gradient in virus gene expression, resulting in reduced levels of 'M_1' protein expression in infected cells and thereby reducing the rate of virus assembly and maturation. Other genes and/or regulatory regions may also be translocated appropriately, in some cases from other strains of RSV of human or animal origin. For example, the 'F' gene (and possibly the 'G' gene) of a human subgroup B RSV could be inserted into an otherwise RSV strain A genome (in place of, or in addition to the RSV strain A 'F' and 'G' genes).

In another approach, the RNA sequence of the RSV viruses 'N' protein is translocated from its 3' proximal site to a position closer to the 5' end of the genome, again taking advantage of the 3' to 5' gradient in gene transcription to reduce the level of N protein produced. By reducing the level of N protein produced, there would result a concomitant increase in the relative rates of transcription of genes involved in stimulating host immunity to RSV and a concomitant reduction in the relative rate of genome replication. Thus, by translocating the RSV RNA sequence coding for RSV N protein, a chimeric RSV virus having attenuated pathogenicity relative to native RSV will be produced.

Another exemplary translocation modification resulting in, in effect, the introduction of heterologous sequence as is discussed herein may result in the production of attenuated chimeric RSV comprises the translocation of RSV RNA sequence coding for the 'L' protein of RSV. This sequence of the RSV virus is believed responsible for viral polymerase protein production. By translocating the RSV sequence coding for L protein from its native 5' terminal location in the native RSV genome to a location at or near the 3' terminus of the genome, a chimeric RSV virus exhibiting attenuated pathogenicity will be produced. Yet another exemplary translocation comprises the switching the locations of the RSV RNA sequences coding for the RSV G and F proteins (ie, relative to each other in the genome) to achieve a chimeric RSV having attenuated pathogenicity resulting from the slight modification in the amount of the G and F proteins produced. Such gene shuffling modifications as are exemplified and discussed above are believed to result in a chimeric, modified RSV having attenuated pathogenicity in comparison to the native RSV starting material. The nucleotide sequences for the foregoing encoded proteins are known, as is the nucleotide sequence for the entire RSV genome. See McIntosh, Respiratory Syncytial Virus in Virology, 2d Ed. edited by B. N. Fields, D. M. Knipe et al., Raven Press, Ltd. New York, 1990 Chapter 38, pp1045–1073, and references cited therein.

These modifications can additionally or alternatively comprise localized, site specific, single or multiple, nucleotide substitutions, deletions or additions within genes and/or regulatory domains of the RSV genome. Such site specific, single or multiple, substitutions, deletions or additions can reduce virus pathogenicity without overly attenuating it, for example, by reducing the number of lysine or arginine residues at the cleavage site in the F protein to reduce efficiency of its cleavage by host cell proteases (where cleavage is believed to be an essential step in functional activation of the 'F' protein), and thereby possibly reduce virulence. Site specific modifications in the 3' or 5' regulatory regions of the RSV genome may also be used to increase transcription at the expense of genome replication. In addition, localized manipulation of domains within the N protein, which is believed to control the switch between transcription and replication can be made to reduce genome replication but still allow high levels of transcription. Further, the cytoplasmic domain(s) of the G and F glycoproteins can be altered in order to reduce their rate of migration through the endoplasmic reticulum and golgi of infected cells, thereby slowing virus maturation. In such cases, it may be sufficient to modify the migration of G protein only, which would then allow additional up-regulation of 'F' production, the main antigen involved in stimulating neutralizing antibody production during RSV infections. Such localized substitutions, deletions or additions within genes and /or regulatory domains of the RSV genome are believed to result in chimeric, modified RSV also having reduced pathogenicity relative to the native RSV genome starting material.

9.3 Use of Monoclonal Antibodies to Differentiate Rescued Virus from Helper Virus

In order to neutralize the RSV strain B9320 helper virus and facilitate identification of rescued A2 strain RSV, monoclonal antibodies against RSV strain B9320 were made as follows.

Six BALB/c female mice were infected intranasally (i.n.) with ~10^5 plaque forming units (p.f.u.) of RSV B9320, followed 5 weeks later by intraperitoneal (i.p.) inoculation with 10^6–10^7 pfu of RSV B9320 in a mixture containing 50% complete Freund's adjuvant. Two weeks after i.p. inoculation, a blood sample from each mouse was tested for the presence of RSV specific antibody using a standard neutralization assay (Beeler and Coelingh, *J. Virol.* 63:2941–2950 (1988)). Mice producing the highest level of neutralizing antibody were then further boosted with ~10^6 p.f.u. of RSV strain B9320 in phosphate buffered saline (PBS), injected intravenously at the base of the tail. Three days later, the mice were sacrificed and their spleens collected as a source of monoclonal antibody producing B-cells. Spleenocytes (including B-cells) were teased from the mouse spleen through incisions made in the spleen capsule into 5 ml of Dulbecco's Modified Eagle's Medium (DME). Clumps of cells were allowed to settle out, and the remaining suspended cells were separately collected by centrifugation at ~2000×g for 5 minutes at room temperature. These cell pellets were resuspended in 15 ml 0.83% (w/v) NH_4Cl, and allowed to stand for 5 minutes to lyse red

blood cells. Spleenocytes were then collected by centrifugation as before through a 10 ml cushion of fetal calf serum. The spleenocytes were then rinsed in DME, repelleted and finally resuspended in 20 ml of fresh DME. These spleenocytes were then mixed with Sp2/0 cells (a mouse myeloma cell line used as fusion partners for the immortalization of spleenocytes) in a ratio of ~10:1, spleen cells: Sp2/0 cells. Sp2/0 cells were obtained from the ATCC and maintained in DME supplemented with 10% fetal bovine serum. The cell mixture was then centrifuged for 8 minutes at 2000×g at room temperature. The cell pellet was resuspended in 1 ml of 50% polyethylene glycol 1000 mol. wt. (PEG 1000), followed by addition of equal volumes of DME at 1 minute intervals until a final volume of 25 ml was attained. The fused cells were then pelleted as before and resuspended at 3.5×10^6 spleen cells ml^{-1} in growth medium (50% conditioned medium from Sp2/0 cells, 50% HAT medium containing 100 ml RPMI 25 ml F.C.S., 100 μgml^{-1} gentamicin, 4 ml 50× Hypoxanthine, Thymidine, Aminopterin (HAT) medium supplied as a prepared mixture by Sigma Chem. Co., St. Louis, Mo.). The cell suspension was distributed over 96 well plates (200 μl $well^{-1}$) and incubated at 37° C., 95% humidity and 5% CO_2. Colonies of hybridoma cells (fused spleenocytes and Sp2/0 cells) were then subcultured into 24 well plates and grown until nearly confluent; the supernatant growth medium was then sampled for the presence of RSV strain B9320 neutralizing monoclonal antibody, using a standard neutralization assay (Beeler and Coelingh, *J. Virol.* 63:2941–50 (1988)). Hybridoma cells from wells with neutralizing activity were resuspended in growth medium and diluted to give a cell density of ~0.5 cells per 100 μl and plated out in 96 well plates, 200 μl per well. This procedure ensured the production of monoclones (i.e. hybridoma cell lines derived from a single cell) which were then reassayed for the production of neutralizing monoclonal antibody. Those hybridoma cell lines which produced monoclonal antibody capable of neutralizing RSV strain B9320 but not RSV strain A2 were subsequently injected into mice, i.p. (10^6 cells per mouse). Two weeks after the i.p. injection mouse ascites fluid containing neutralizing monoclonal antibody for RSV strain B9320 was tapped with a 19 gauge needle, and stored at −20° C.

This monoclonal antibody was used to neutralize the RSV strain B9320 helper virus following rescue of RSV strain A2 as described in Section 9.1. This was carried out by diluting neutralizing monoclonal antibody 1 in 50 with molten 0.4% (w/v) agar in Eagle's Minimal Essential Medium (EMEM) containing 1% F.C.S. This mixture was then added to Hep-2 cell monolayers, which had been infected with the progeny of rescue experiments at an m.o.i. of ~0.1–0.01 pfu $cell^{-1}$. The monoclonal antibody in the agar overlay inhibited the growth of RSV strain B9320, but allowed the growth of RSV strain A2, resulting in plaque formation by the A2 strain. These plaques were picked using a pasteur pipette to remove a plug of agar above the plaque and the infected cells within the plaque; the cells and agar plug were resuspended in 2 ml of EMEM, 1 % FCS, and released virus was plaqued again in the presence of monoclonal antibody on a fresh Hep-2 cell monolayer to further purify from helper virus. The twice plaqued virus was then used to infect Hep-2 cells in 24 well plates, and the progeny from that were used to infect 6 well plates at an m.o.i. of ~0.1 pfu $cell^{-1}$. Finally, total infected cell RNA from one well of a 6 well plate was used in an RT/PCR reaction using first and second strand primers on either side of the 'marker sequences' (introduced into the RSV strain A2 genome to act as a means of recognizing rescue events) as described in Section 9.2 above. The DNA produced from the RT/PCR reaction was subsequently digested with StuI and PmeI to positively identify the 'marker sequences' introduced into RSV strain A2 cDNA, and hence to establish the validity of the rescue process.

10. DEPOSIT OF MICROORGANISMS

An *E. coli* cell line containing the plasmid pIVACAT is being deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL; and has the following accession number

Strain	Plasmid	Ascension Number
E. coli (DH5a)	pIVACAT	NRRL

The present investigation is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs, viruses or enzymes which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. 78.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 43

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(i x) SEQUENCE DESCRIPTION: SEQ ID NO:1:

-continued

GAAGCTTAAT ACGACTCACT ATAAGTAGAA ACAAGGGTGT TTTTTCATAT CATTTAAACT 60

TCACCCTGCT TTTGCTGAAT TCATTCTTCT GCAGG 95

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTGCAGAAG AATGA 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGCTTAAT ACGACTCACT ATAAGCAAAA GCAGGGTGAA GTTTAAATGA TATGAAAAAA 60

CACCCTTGTT TCTACTGAAT TCATTCTTCT GCAGG 95

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTTAATAC GACTCACTAT AAGATCTATT AAACTTCACC CTGCTTTTGC TGAATTCATT 60

CTTCTGCA 68

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAAGAATGAA TTCAGCAAAA GCAGGGTGAA GTTTAATAGA TCTTATAGTG AGTCGTATTA 60

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCGAATTCTT AATACGACTC ACTATAAGTA GAAACAAGGG TG 42

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTCTAGACG CTCGAGAGCA AAAGCAGGTG 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACCCUGCUU UUGCU 15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CACCCUGCUU UUACU 15

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACCCUGCUU CUGCU 15

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACCCUGUUU UUGCU 15

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACCCUGUUU UUACU 15

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CACCCUGUUU CUGCU 15

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACCCUUGCU UUUGCU 16

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CACCCUUGCU UUUACU 16

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CACCCUUGUU UUUACU 16

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC RNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCCUUGUU UCUACU 16

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTAGACGCCC TGCAGCAAAA GCAGGGTGAC AAAGACATAA TGGAGAAAAA AATCACTGGG 60

TATACCACCG TTGATATATC CCAATCGCAT CGTAAA 96

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGCGGGACGT CGTTTTCGTC CCACTGTTTC TGTATTACCT CTTTTTTTAG TGACCCATAT 60

GGTGGCAACT ATATAGGGTT AGCGTAGCAT TTCTTG 96

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

actgcgatga gtggcagggc ggggcgtaat agat 34

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

tgacgctact caccgtcccg ccccgcatta tctagatc 38

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACTGCGATGA GTGGCAGGGC GGGGCGTAAT AGAT 34

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTAGATCTAT TACGCCCCGC CCTGCCACTC ATCGCAGT 38

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 97 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTAGACGCCC TGCAGCAAAA GCAGGGTGAC AAAGACATAA TGGAGAAAAA AAATCACTGG 60

GTATACCACC GTTGATATAT CCCAATCGCA TCGTAAA 97

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTTCTTTACG ATGCGATTGG GATTTTGCTG CAGGGCGT 38

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CGGAATTCTC TTCGAGCGAA AGCAGGAGTT 30

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCAAGCTTAT TAACCCTCAC TAAAAGTAGA AACAAGGAGT TT 42

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CATGGGTGAG TTTCGACCAA AATCTAGATT ATAAAATAGG ATACATATGC A 51

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AATGTATCCT ATTTTATAAT CTAGATTTTG GTCGAAACTC ACC 43

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGACGCATAT TACGCGAAAA AATGCGTACA ACAAACTTGC ATAAAC 46

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAAAAAAATG GGCAAATAA GAATTTGATA AGTACCACTT AAATTTAACT 50

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTAGAGTTAA ATTTAAGTGG TACT 24

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATCAAATTC TTATTTGCCC CATTTTTTTG GTTTATGCAA GTTTGTTGTA 50

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CGCATTTTTT CGCGTAATAT GCGTCGGTAC 30

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTATTCAATT ATAGTTATTA AAAATTAAAA ATCATATAAT TTTTTAAATA 50

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ACTTTTAGTG AACTAATCCT AAAGTTATCA TTTTAATCTT GGAGGAATAA 50

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ATTTAAACCC TAATCTAATT GGTTTATATG TGTATTAACT AAATTACGAG 50

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 bases
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATATTAGTTT TTGACACTTT TTTTCTCGTT ATAGTGAGTC GTATTA 46

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGCTTAATAC GACTCACTAT AACGA 25

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GAAAAAAAGT GTCAAAAACT AATATCTCGT AATTTAGTTA ATACACATAT 50

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAACCAATTA GATTAGGGTT TAAATTTATT CCTCCAAGAT TAAAATGATA 50

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ACTTTAGGAT TAGTTCACTA AAAGTTATTT AAAAAATTAT ATGATTTTTA 50

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: SYNTHETIC DNA

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATTTTTAATA ACTATAATTG AATACTGCA 29

What is claimed is:

1. A recombinant RNA molecule comprising a binding site specific for an RNA-directed RNA polymerase derived from a respiratory syncytial virus, operatively linked to a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence.

2. A recombinant RNA molecule comprising a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a 3'-noncoding viral sense flanking sequence of a respiratory syncytial virus containing the viral polymerase binding site, and to a 5**40** -noncoding viral sense flanking sequence of respiratory syncytial virus.

3. A recombinant RNP comprising the recombinant RNA molecule of claim **1** mixed with the purified RNA-directed RNA polymerase.

4. A recombinant RNP comprising the recombinant RNA molecule of claim **3** mixed with purified respiratory syncytial viral polymerase.

5. A recombinant RNP comprising the recombinant RNA molecule of claim **2** mixed with purified respiratory syncytial viral polymerase.

6. A chimeric virus comprising a respiratory syncytial virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a respiratory syncytial viral polymerase binding site.

7. A chimeric virus comprising a respiratory syncytial virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a polymerase binding site of the respiratory syncytial virus.

8. A method for producing a chimeric respiratory syncytial virus (RSV) comprising:

(a) culturing a host cell transfected with a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence operatively linked to a RSV polymerase binding site and infected with a parental strain of RSV and,

(b) recovering said chimeric virus from the culture.

9. A chimeric RSV produced by the method of claim **8**.

* * * * *

Book 1

LABORATORY
NOTEBOOK

DAVID K. CLARKE

AVERON

B000001

reference binder #22A

NOTEBOOK NO. 1

ISSUED TO DAVID K. CLARKE.

ON

DEPARTMENT AVIRON.

RETURNED

—SCIENTIFIC NOTEBOOK CO.—
2831 LAWRENCE AVE.
P.O. BOX 238
STEVENSVILLE, MI 49127
616-429-8285

INSTRUCTIONS

1. **The primary purpose of this notebook is to protect your and the Company's Patent-Rights by keeping records of all original work in a form acceptable as evidence if any legal conflict arises.**

2. • When starting a page, enter the title, project number, and book number.
   • Use <u>ink</u> for permanence -- avoid pencil.
   • Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
   • Avoid making notes on loose paper to be recopied.
   • Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.

3. • Give a complete account of your experiments and the results, both positive and negative, including your observations.
   • Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
   • Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.

4. • After entering your data, sign and date the entries.
   • Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
   • Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.

5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.

6. This notebook and its contents are the exclusive property of the Company. It is confidential and the contents are not to be disclosed to anyone unless authorized by the Company. You must return it when completed, upon request, or upon termination of employment. It should be kept in a protected place. **If loss occurs, notify your supervisor immediately, and make a written report describing the circumstances of the loss.**

Form 590

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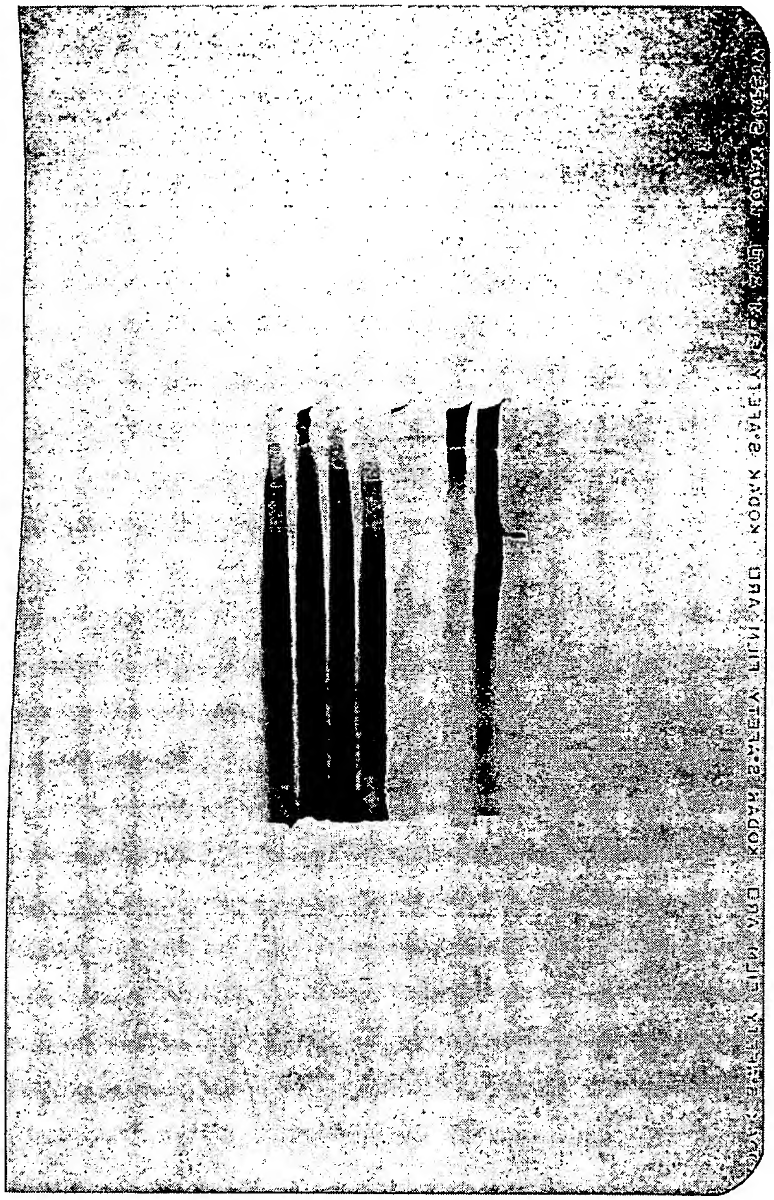
‡00B

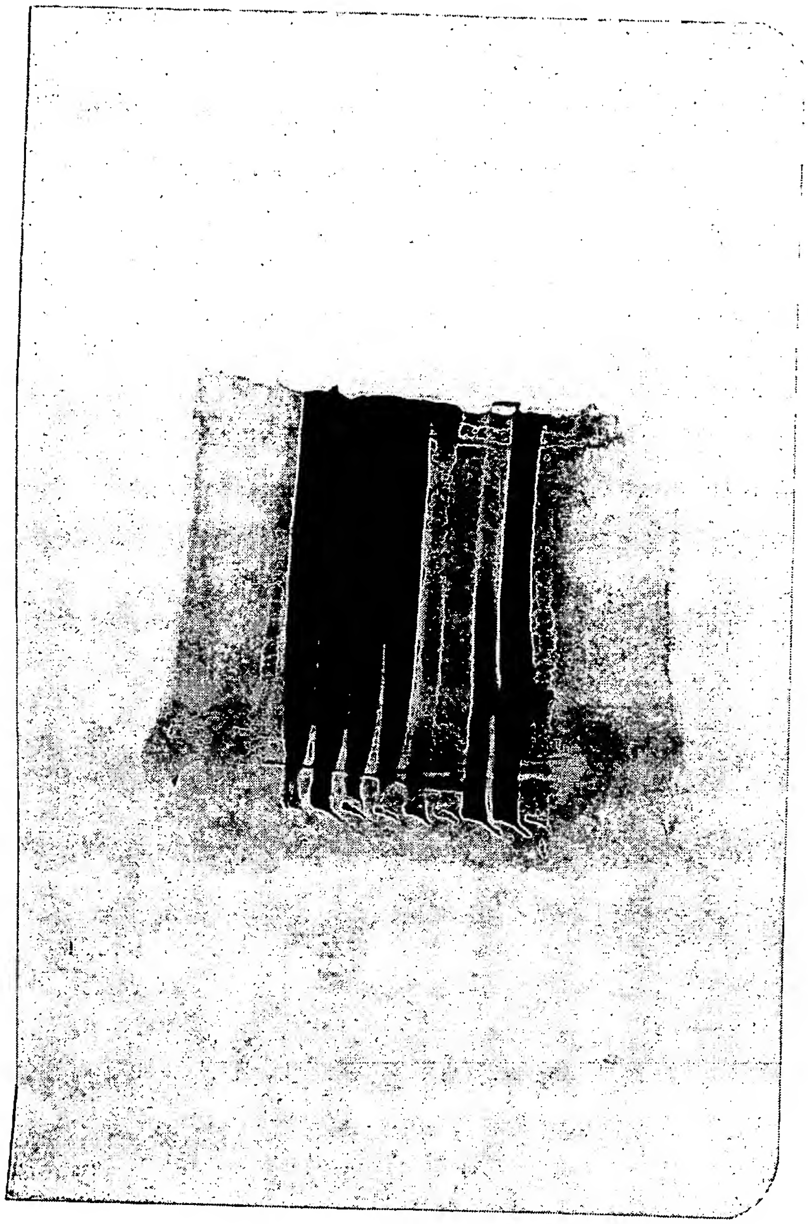
293 cells

.HEADLINE
ATCC CRL-1573 293 (Transformed primary embryonal kidney, human)
.TEXT
Passage Frozen: 31. Current medium for propagation: Eagle's MEM with Earle's BSS, 90%; heat-inactivated horse serum, 10%. Additional Information: The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and contain and express the transforming genes of Ad 5. Handle as potentially biohazardous material under at least Biosafety Level 2 containment. The line has been used in the isolation of transformation defective, host-range mutants of Ad 5, and is excellent for titrating human adenoviruses. This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells. References: J. Gen. Virol. 36: 59-72, 1977; Virology 77: 319-329, 1977; ibid., 86: 10-21, 1978. Submitted by: F.L. Graham, McMaster University, Hamilton, Ontario, Canada.
Price Code: J

Aye W. Khl



[redacted]000022



TITLE: RSV VACCINE DEVELOPMENT.

Project No.

Book No.

From Page No.

1st Week at Aviron was spent meeting people, finding out how the various support systems operated, getting a desk/office organised, and reading background material on RSV, and Herpes Viruses.

Looked over protocol for RSV growth and storage. Identified RSV subgroups (A) and (B) in Kathy's freezer box.

Prepared EMEM supplemented with

(A) = pp 3D A2 HEP 4.

(B) = pp3 18537 HEP 4

(1) Glutamine (1% v/v - stock 29.3g L^{-1})

(2) Streptomycin, Penicillin G } 1% v/v - stock 25g L^{-1} - stock 15.77g L^{-1}

(3) F.C.S. 1% heat inactivated.

Infected 2 x 75cm^2 monolayers of Vero cells, each with ~~20~~ 0.5ml ($\approx 10^6$ pfu ml^{-1}) of RSV. Used 2 Flasks for subtype A and 2 Flasks for subtype B.

Adsorbed virus on monolayer for 1-2 hrs - then added EMEM (10ml ~~10-20ml~~) and continued incubation to observe C.P.E.

Prepared separate stocks of 1M Hepes and 2M $MgSO_4$ (100ml). Autoclaved and stored in tissue culture room.

Checked cytopathic effect of A2 and 18537 on Vero cell (under phase contrast). There appears to be some cytopathic effect, apparent at localised regions of the monolayers. The main effect appears as a fusion of cell membranes to form syncytia.

To Page No. 2

Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke

Project No.________

Book No.________ TITLE ________

From Page No. 1

Need to order in the future.

1. Phenol chloroform*
2. Methyl Mercury*
3. Taq. Polymerase — * Immediately.
4. Reverse Transcriptase
5. Mechanical stirrers* ✓
6. HEp-2 cells* ✓

Reverse Transcriptase should be Avian from Life Sciences St. Petersburg, Florida
1-800-237 4323
CATALOG No. AZM-007.
1000u/vial.

Prepared stock solutions of 20% SDS (ULTRA PURE)
Prepared a stock solution of 1M $MgCl_2$
Autoclaved along with a Deionised H_2O supply.

---

am. Checked cytopathic effect on Vero cells again. Noted extensive c.p.e with A2 in both flasks. The c.p.e was evident as large areas of fused cells which also looked v. sickly and 'grainy'. Veros infected with sub-type 'B' showed only v. limited and localised c.p.e with small areas of fusion – will check both again later today.

Ordering info: Magnetic Stirrer.
ordered — E and K Scientific Products, Inc.
CAT No. 500P Price $185
Received

AVIAN REV. TRANSCRIPTASE
Life Sciences Inc.
2900 72nd STREET NORTH.
St. Petersburg
Florida 33710.
Fax (813) 347-2957.
1000u – $125 + $35 freight

SATURATED PHENOL/CHLOROFORM
AMRESCO.
received — Product Code 0945
100mL $25-80.

arrives O/N on Dry Ice.
No shipping on Fri(s).

To Page No. 2

Witnessed & Understood by me,	Date	Invented by	Date
Holly Abrams			

Recorded by
DAVID K. CLARKE

Project No.

TITLE: Book No.

From Page No. 2

Proposed scheme for utilisation of virus stocks.

A2 and 18537 (1 ml vials from NIH).

½ml / Flask 75cm² ½ml / Flask.

20ml 20ml

Harvested and used as stock | Harvested and used as stock (Label carefully).

- Try new cell lines.
- Try different M.O.I.s. < 1ml.
- Try different incubatn temp.

Preparation of working stocks. (200-400ml). | Preparation of working stocks. (200-400ml).

ATCC ACC. N° 117BS

≈ 100 Roller bottles worth. " " " "

Ordered. HEp-2 cells 2 x 1 ml vials at ≈ $97-w vial. (ATCC)

4-30pm initiated Harvest of RSV A2 due to advanced c.p.e. Followed protocol.

1) Scrapped infected cells into supernatant.
2) centrifuged 10mins at 4°C 3,000 rpm.
3) Decanted super. into clean tube - resuspended pellet in 2ml fresh EMEM, and went through freeze-thaw cycle - repelleted and pooled super with 1st - Added 1ml 1M Hepes, 1ml of 2M $MgSO_4$ (noted v. alkaline pH - adjusted with conc. HCL) Aliquotted virus to 1ml vials - froze in dry-ethanol ice bath. Then stored virus at -70°C. This stock was named RSV A2 P_1 Vero + DATED.

To Page No. 4

Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke.

Project No.______
Book No.______ TITLE ______________________

From Page No. 3

Checked c.p.e. on 18537 infected cells — there were numerous localised areas of multicellular syncytial formation, but not sufficiently widespread to warrant harvesting. Poured off old medium and added 10ml fresh medium to each flask, in an attempt to stimulate virus production – will check again this afternoon and tomorrow morning.

Located supplier of Methyl mercury II hydroxide

Received
Stored in Fume hood Dave's/Neils lab

Alpha-Aesar – Fax 1-800-322-4757 Tel. 1-800 343 1990

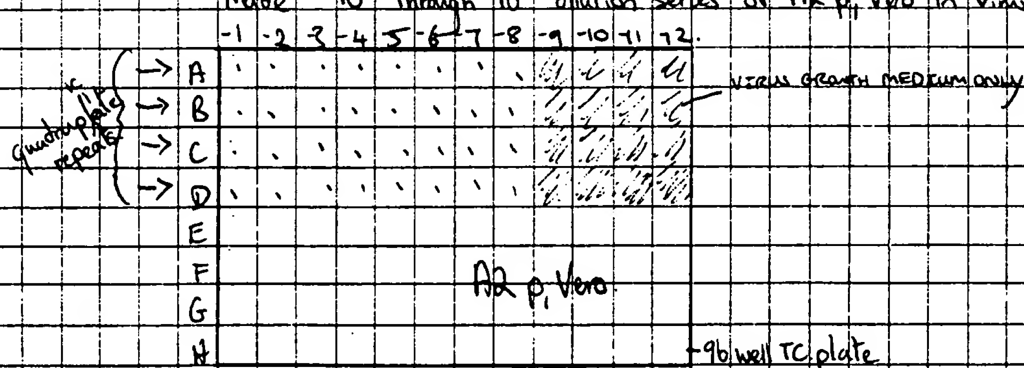
Fed I.D N° Required
Book Ref

CAT. N° 13395 50mL ≡ $295

Ordered Phenol chloroform from Amresco and and Methyl mercury from Aesar. Received Both

Set up $TCID_{50}$ Quantitation assay on RSV stock A2 P_1 Vero grown up yesterday.

Made 10^{-1} through 10^{-8} dilution series of A2 p_1 Vero in Virus growth medium



Empty out growth medium Replace with 200µl of diluted virus.

Calculation of titer. 1 well with c.p.e ≡ 0.5 log of virus/100µl
May be inaccurate due to variations in cell density.

2 wells ≡ 1 log
3 wells ≡ 1.25 logs
4 wells ≡ 1.5 logs.

i.e. after 2 wells each well is worth 0.25 logs of virus

To Page No. 5

Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date
Recorded by David K Clarke

Project No.

TITLE: Book No.

From Page No. 4

Checked cytopathic effect of 18537 virus on vero — there is much more fusion and giant cell formation than yesterday — the 'trick' of adding fresh medium seems to have worked!

Also checked 96 well plate for $TCID_{50}$ Results — Not yet clear, but it seems that the 10^{-1} — 10^{-3} dilutions are showing significant fusion activity — will leave over the weekend and do final read on Monday or Tuesday. Will check c.p.e on 18537 later this afternoon and decide if should harvest today or tomorrow.

Prepared a fresh 1M Hepes solution (pH 7.5 with HCl) — autoclaved in 'big autoclave.' Stored in Tissue culture room.

Checked c.p.e. (4pm) — progressing well — will harvest either later tonight (after dinner with Pim+K) or tomorrow. $TCID_{50}$ looks pretty good — at least 10^{-1} and 10^{-2} dilutions are showing complete fusogenic activity — may indicate a titre of (assuming 10^3 cells/well) $\geq 10^4$–10^5 pfu.ml^{-1} Will read again tomorrow and Monday

Ordered Methyl Cellulose from VWR 1Kg MX08501 received: Stored in Chemicals Rm.

6pm checked c.p.e again — will leave untill tomorrow to harvest.
Will also check $TCID_{50}$ plate.

Harvested RSV 18537 p1 Vero — c.p.e was not as advanced as I would have liked, but it would have been counterproductive to wait any longer
Added 1ml of 1M Hepes pH 7.5 + 1ml of 2M $MgSO_4$ — Aliquotted and froze in

EtOH/dry ice — then put at -70°C

Checked $TCID_{50}$ plate — Now looks as though there is c.p.e 10^{-1} — 10^{-3}
Will check again on Mon 5 days post infection.

To Page No. 6

Witnessed & Understood by me,	Date	Invented by	Date
Holly Abrams		Recorded by David K Clarke	

000028

From Page No. 5

Checked TCID$_{50}$ plates (Day 5 post infection). Results look very promising - approx $10^{7.5}$ pfu ml^{-1} (or greater!) Will make final reading tomorrow.

Tomorrow will carry out TCID$_{50}$ measurement on RSV 18537 $10^{-1} - 10^{-8}$

(RSV 18537 p, Vero) Will also try to boost growth of 18537 (would predict that titer will be low). To do this will infect 1 x 25 cm^2 flask with 0.5ml of undilute virus and one flask with 0.5ml of 10^{-1} diluted virus. - Adsorb 2hrs+

(Good for 75 cm^2 bottle) Similarly - will infect 1 x 25 cm^2 bottle with ≈ 200µl of undilute RSV A2 p, Vero and 1 x 25 cm^2 bottle with 200µl of dilute (10^{-1}) RSV A2 p, Vero.

Received Stored in chem stocks. Ordered 100g Sigma Hepes (as a highly pure stock for careful mol. Biol. work)

Organised a scheme for synthesising a full length cDNA for the RSV Genome

Checked RSV A2 P, Vero TCID$_{50}$ -

27 TOTAL SHOWING C.P.E

$1 + \frac{25}{4} = 7.25$

i.e titer of stock is $10^{7.25}$ ml^{-1} (Pretty damn good!)

Started infection of small 25 cm^2 (?) bottles

Used undilute and 10^{-1} diluted inoculum for RSV A2 and RSV 18537 P, Vero.

Used 0.5ml of each dilution for RSV 18537 - in an attempt to boost M.O.I and build up titer of that stock.

Used 0.2ml of each dilution for RSV A2 p, Vero in an attempt to build

To Page No. 7

Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke

Judy Beeler 9-1-(301) 402-0414

Project No.

TITLE

Book No.

1% fungizone

From Page No. 6

up titers even more for that stock, possibly by eliminating DI at 10^{-1} or increasing MOI at undilute.

Invitrogen called about T/A vector – They said it should be possible to PCR up 5Kb fragments and clone them.

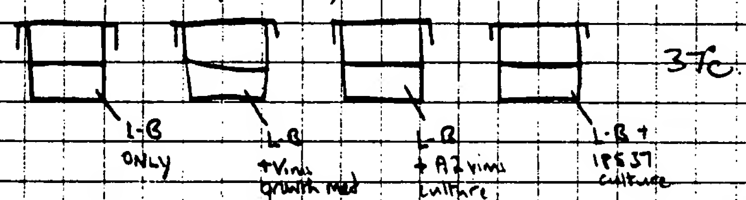
After adsorbing virus for ≈2hrs at 37°C added 3-4ml of virus growth medium and replaced at 37°C.

≈3-30pm Set up TCID$_{50}$ on RSV 18537 p, Vero 10^{-1} — 10^{-8} dilutions. Incubated o/n to observe c.p.e. will check again daily through the weekend.

Checked c.p.e. on cell monolayers infected with RSV. – 24h p.i. C.p.e. is not yet distinctive - will check again later this afternoon.

The TCID$_{50}$ is not showing generalised fusion (as the A2 did) at 10^{-1} and 10^{-2} – indicating the input titer is v. low.

Will set up sterility tests on virus cultures + virus growth medium.



3-45pm Checked c.p.e. again on A2 and 18537 infected cells – There is advanced c.p.e. on the undilute - infected cells; and quite a large amount of c.p.e on the A2 10^{-1} infected cells. There was no observeable c.p.e on the 10^{-1} dil 18537 infected cells, but perhaps a little at undil 18537. Nothing is visible yet on the TCID$_{50}$ 18537 plate.

NB Should try different cell lines for RSV 18537 if yields cannot be improved on Vero.

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Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke

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Checked sterility tests - they are all negative (Excellent). The gritty particulate material in the culture medium must be the result of virus cytopathology.

Checked c.p.e on Infected monolayers. The two A2 infected monolayers have been wiped out (10^{-1} + undilute). The 18537 monolayers were also showing cytopathic effect at both dilutions - will incubate them for a further 24hrs. Will harvest the A2 virus this morning.

Harvested A2 virus - separately for each dilution - observed supernatants for presence of any possible contaminants (see above) - none were observed.

$TCID_{50}$ not yet showing any appreciable c.p.e!

Left Message with Judy Beeler for some more vials of RSV (Lyn Booth, Susi, or other possible contacts)

Prepared fresh 10% (w/v) S.D.S - autoclaved - stored in mol. biol. lab.

Final observation of c.p.e. on 18537 infected cells for the day; looks good at both dilutions - will probably harvest tomorrow.

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10% SDS solution has turned milky; Maybe autoclaving is not useful for high percentage SDS solns!!

Checked $TCID_{50}$ (RSV 18537 p2 Vero) - c.p.e now detectable out to 10^{-3} dilution after 3 days; will make final reading on monday.

6hpi Checked c.p.e on RSV 18537 p_1 Vero infected cells — Undil - shows massive c.p.e.
dil - shows good c.p.e. but not as extensive as the 'undil' flask.

Sterility 'control' is still the only one which shows any sign of contamination. This indicates that fortuitous contamination can occur; however it appears that none of my virus stocks or solutions are contaminated.

Initiated harvest of virus from RSV 18537 P_2 Vero undil and 10^{-1} dil^n followed protocol carefully - froze away in 0.5ml or 1ml aliquots as RSV 18537 P_2 Vero 10^{-1} dil or Undil.

Carried out $TCID_{50}$ on RSV A2 P2 Vero 10^{-1} undil. + RSV 18537 P_2 Vero 10^{-1} undil.

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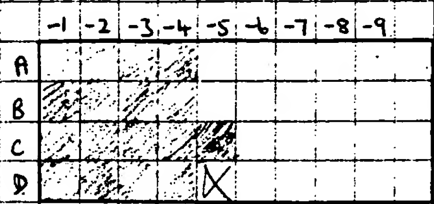
Came in to check on on TCID$_{50s}$

① Day 5 post infection for RSV 18537 p_1 Vero (Final reading tomorrow unlikely to change).



17 wells showing c.p.e ≡ 1+3.75 = $10^{4.75}$

This value is close to the expected titer

② Also checked TCID$_{50s}$ (at 48hpi) for A2 p_2 Vero 10^{-1}/undil and 18537 p_2 Vero 10^{-1} and undilute. It appears that for both viruses cpe is occurring more rapidly and to a greater extent than at the p_1 passages - indicating either/or increased titers and improved growth efficiency (Final reading for these plates will be wed/thur).

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Did final Read on $TCID_{50}$ for RSV 18537 P_1 Vero and did 3 day check on RSV A2 P_2 Vero 10^{-1}/undil and RSV 18537 P_2 Vero 10^{-1}/undil.

(6 day) Final reading on 18537 P_1 Vero = 18 wells showing c.p.e.

titer = 10^5 ml^{-1}

RSV A2 P_2 Vero – already showing good c.p.e. out to $10^{-6}/10^{-7}$ at both dilutions.

RSV 18537 P_2 Vero – showing c.p.e. out to $10^{-3}/10^{-4}$ at both dilutions. (Perhaps a little more extensive at the same time as for 18537 P_1 Vero) Will check again this evening.

Ordered 2 small $25cm^2$ bottles for tomorrow, $10 \times 75cm^2$ bottles for Wed and 2 x 96 well plates for Friday.

Ordered 1 TA cloning kit (20 RXN - #245) InVitrogen

Ordered Taq Polymerase 100u Stratagene ~~received~~ - stored at -20°C

Ordered RNase/DNase free Sucrose Sigma 1Kg Received - store room.

Checked $TCID_{50}$s once again - it appears that A2 P_2 Vero undil is showing extensive c.p.e. out to 10^{-8} dilution which is a titer of $10^{8.5}$
The diluted A2 P_2 Vero is not quite as extensive

The 18537 P_2 Vero undil were showing c.p.e to 10^{-4} – will check both again tomorrow (Day 4)

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Recorded by Daniel K Clarke

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Checked $TCID_{50}$s again – There is c.p.e at 10^{-4} dilution on RSV A2 p2 Vero Undil with a total of 31 wells showing c.p.e. This is equivalent to a titer of $10^{3.25}$ It would appear that this pool is devoid of DI's so far, as titer is increasing with increasing M.O.I.

The RSV 18537 p2 Vero $TCID_{50}$s are showing some c.p.e. out to 10^{-5} dilution in the 'DIL' infection but NOT at 'UNDIL' infection – indicating a presence of DI particles in this stock. If c.pe. occurs at 10^{-5} in all 4 wells then a titer of $10^{5.5}$ would be indicated.

11am. Infected two $25cm^2$ flasks (≈100% confluent) with RSV 18537 p2 Vero [redacted]ndil. (from $TCID_{50}$ this stock should be higher titer than 'UNDIL' stock) Did one infection with 0.5ml of this stock undiluted, and for the 2nd flask infected with 50µl of undilute stock + 150µl MEM (200µl final vol) to give equivalence in pfu to infecting with 0.5ml of 100µl + 900µl (10^{-1} dil^n). This reduction in volume may facilitate adsorption while minimizing DI involvement.

Completed infection process at 1-45pm – added 3.5ml MEM to undil infection and 4.0ml to 10^{-1} dil^n infection.

Checked $TCID_{50}$s again. May have jumped the gun on 18537. C.p.e. may be better on undil. Will wait another day or two to find out. Meanwhile will monitor dil infections set up earlier this morning.

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Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David R Clarke

[redacted]000035

From Page No.__

Checked Vero cells needed for production of working stock - they are not yet sufficiently confluent - will check again at ≈ 3pm but most probably will wait until tomorrow to infect them.

Checked $TCID_{50}$s once more :- it seems that 10^{-1} dil^n RSV 18537 p2 Vero is giving the highest titer (vs. undil A2 p2 Vero) - out to $10^{-5} = 10^{5.5} ml^{-1}$ (up ½ log). Monolayers infected with 18537 p2 Vero Dil (at undil + dil 10^{-1}) are already showing a little c.p.e at 24h.p.i. especially at 10^{-1} dilution

RSV A2 p2 Vero $TCID_{50}$ is showing good c.p.e - especially at Undil where a titer of $10^{8.25}$ is indicated (31 wells with c.p.e.) - up ≈ 1 log!!

Taq Pol arrived from Stratagene.

Checked confluence of Veros for working stock of A2 - still not ready - will wait until tomorrow to infect to ensure max. virus production.

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Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke

B000036

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From Page No.__

Did final Read on $TCID_{50s}$ for RSV A2 P_2 Vero undil/dil – Undil gave highest titer

at

	-1	-2	-3	-4	-5	-6	-7	-8
A								
B								
C								
D								

31 wells with c.p.e. ≡ 1+7 = $\boxed{10^{8.25}}$

undil.

For RSV 18537 P_2 Vero

	-1	-2	-3	-4	-5
A					
B					
C					
D					

Dil – gave highest titer

20 wells with c.p.e.
Translates into titer of 4+1 ≡ 5

titer ≡ $10^{5.5}$

Checked c.p.e. on RSV 18537 p2 Vero dil – infected cells – they are progressing well at both 10^{-1} dil" and undil. ~ will probably harvest tomorrow

Checked confluence of cells to be infected with RSV A2 P_1 Vero (undil) – They look ready ≈ 95% confluent. Will infect ≈ 2-3pm.

2-45pm Infected 10 x 75 cm^2 Vero monolayers with 0.5-0.7ml of RSV A2 P_1 Vero stock (undiluted) – adsorbed for ≈ 2 hrs at 37°c then fed each monolayer with ≈ 10ml of pre-warmed virus growth medium.

C.P.E. is progressing well on RSV 18537 P_2 Vero dil. infected at 10^{-1} and Undil. Will harvest both tomorrow and do $TCID_{50}$

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Checked CPE on 25cm² bottles infected with RSV 18537 p2 Vero dil/undil. Undil was showing v. extensive c.p.e. Harvested both monolayers (harvesting may not have been as efficient as usual due to car-door panic!!) Aliquotted and froze away. Will do $TCID_{50}$ on dil/undil later today.

Checked c.p.e. on A2 RSV infected cells (had used A2 p, vero undil to prepare stock) Not extensive c.p.e. showing yet, will check again this afternoon.

Set up a 2nd sterility control: this time used T. culture medium in 25cm² flasks, added 5ml of V. growth medium, and a drop of supernatant from RSV A2 infected cells.

Checked through CV's to find a potential RA. — there are ≈ 8 promising individuals — Talked to Kathy, decided not to hire an RA for a month or two (or 3 or 4!!)

Set up $TCID_{50}$ on RSV 18537 $10^{-1} - 10^{-7}$ Dil and Undil
P3 Vero stock.

Prepared 1 litre of STE pH ≈ 7 (100mM NaCl, 10mM Tris, 1mM EDTA) — Autoclaved in the Tomy

35ml Ultraclear tubes for sucrose gradients; probably ≈ 5ml of 60% cushion + 30-33ml of 30%

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Harvested large working stock of RSV A2 P_2 Vero – collected a total of ≈ 100ml. Aliquotted in various sized tubes – froze in EtOH/Dry ice and stored at −90°C

Checked sterility test – does not appear to be anything growing.

It seems that the virus infected cells are extruding cellular material into the medium; it appears to be somewhat attached to adjacent cells and not free floating

Will use working stock diluted 1/2 to make RNA (genomic)

Will need a total infecting volume of 20ml – assuming 1ml/150cm² flask
Will use 10ml of working stock diluted in 10ml of virus growth medium (MED)
It may be possible to use 1/10 vol of W stock, but will check this later in the week

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Checked sterility control - it does appear there is a contaminant present in RSV A2 stock - will try spinning it out with the microfuge - prior to preparation of RNA (genomic)

Checked TCID$_{50}$ (Day 3) for RSV 18537 - There is c.p.e. out to 10^{-4} in 10^{-1} dilution (but not at Undil). Need to check passage Number of Vero's with Kim (They may becoming refractile for RSV.)

Decided to throw out all RSV A2 stocks due to Yeast contamination.

(Called Judy Beeler for more RSV)

Ordered 1ml of RSV A2 from ATCC.

Will infect [redacted] 25cm² Flasks with RSV 18537 - 10^{-1} / P3 Vero Dil - Undil } will try removing supernatant after ≈ 24hrs - 28hrs i.e. ≈ 4-5pm tomorrow

Used 0.5ml for each infection.

Absorbed about 2hrs - fed each with 4.5ml of MEM.

prepared 60% and 30% Sucrose solutions in S.T.E - Boiled for 15-20mins to sterilise - stored at Room temp.

Went over talk with Kathy.

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Checked $TCID_{50}$s for 18537 p_3 Vero dil → Undil → Dil.

Cpe is observable out to 10^{-5} in 2/4 wells at both Dil and Undil passage.

This would give a tentative titer of $\approx 10^5 ml^{-1}$ Down a little from the previous titer, but acceptable as part of the overall process of building up the titer.

Checked cpe on 18537 p_3 vero infected cells 10^{-1} and Undil - nothing significant yet - will change medium around 4-5pm this evening.

Ordered 250 5ml cryovials. (sigma)

Recommendations for PCR if problems arise à la Laurent Roux.

1. Long Ramp time between hybridisat^n and extension was crucial factor.
2. 10% DMSO in Taq. buffer.
3. 16u 100μl^{-1} Taq. Pol. pH? Mg?
4. Fairly long denaturation, annealing and RXN times.

T7 prefers to start an RNA chain with a G but A works too.

✱ Laurent transfected in plasmids containing DI construct (linearised with BamH I) also, NP, P/C and L genes into vaccinia infected cells. Helper virus did not work.

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3pm. Removed virus growth medium from "18537 p_3 Vero Dil" infected cells (10^{-1} and Undil) and replaced with 4ml of fresh virus growth medium (in an attempt to further boost titer of virus stock)

Checked $TCID_{50}$ again - no change — looks like 10^5 will be final titer - but will check again tomorrow and thurs.

Ordered a fridge - freezer (Probably from Sears)

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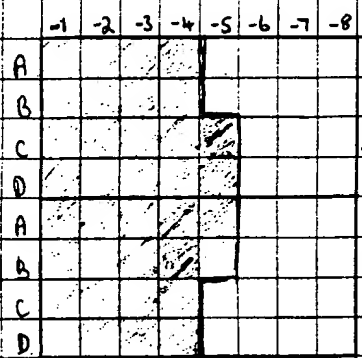
Checked $TCID_{50}$ for RSV 18537 P_3 Vero Dil/Undil stocks (Day 5) Can probably conclude titers today



$10^5 ml^{-1}$ (Dil)

$10^5 ml^{-1}$ (Undil)

This titer is a little down from the P_2 Vero titer; however will continue to passage this virus in an attempt to bring up titer from $10^5 \rightarrow 10^6$

Also checked c.p.e. on '18537 P_3 Vero Dil' - infected cells; 10^{-1} and Undil. C.p.e. is pronounced on both - perhaps a little ahead on the undil infection - might harvest it tonight and 'dil' tomorrow morning.

≈10am Infected 1 x $75cm^2$ of Vero with 0.5ml of RSVA2 P_1 Vero (undiluted) - This virus was saved from the initial growth of RSVA2 prior to addition of $MgSO_4$ / Hepes. I wanted to check if the yeast contamination was present in that stock. If so, it would indicate that the contamination came in with the stock.

Virus arrived from Judy Beeler - stored at -70°C.

2pm Checked c.p.e. again on undil 18537 - Decided to harvest this evening and harvest the dil tomorrow morning.

Stored at -90°C as 18537 P_4 Vero undil.

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Scientific presentations all Day

5pm Harvested RSV 18537 P_4 Vero Dil - aliquotted and froze down at -90°C
C.pe. looked quite advanced.

Scientific Meeting

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Checked c.p.e. on 'A2 p Vero wild' infected Veros - looked advanced, so decided to harvest. Also noticed that the contaminating yeast is still there, indicating that it was probably not introduced by me and probably came in with the stock we received from the FDA, ~~a~~ and in the absence of Fungizone was able to replicate easily.

Notes on Attenuation of RSV :-

① Manipulation of N gene - a) level of expression. - RNA or protein.
b) functional domains - which interacts with RNA.
To give reduced replication, yet good transcription
Has to be sufficient replication to allow rescue.
(May need to use stably transformed cell line to supply N protein in trans, to allow replication of seed vaccine)

② Alter cytoplasmic domains of G and F to give reduced rate of migration through ER and Golgi

③ Eliminate expression of NS1, NS2, M2, SH to observe effects on virus growth and virulence.

④ Mutate M, F and G genes to give slightly reduced expression of these proteins, to slow down maturation

⑤ Reduce number of Lys/Arg residues at cleavage site in F protein to reduce efficiency of cleavage

⑥ Manipulate transcription and and/or replication regulatory sequences.

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(7) Alter part of (M) protein involved in switching off of host transcription - without affecting assembly

Made preparations for $TCID_{50s}$ before 2pm meeting with Peter Palese. Will make dilutions + infections later today

Set up $TCID_{50s}$ on 'A2 p_2 Vero undil'
18537 p_4 Vero dil/undil

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Witnessed & Understood by me, Holly Abrams. HOA
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Day 3. Checked $TCID_{50s}$ – There is c.p.e. out to 10^{-4} in Undil 18537 P_4 Vero
out to 10^{-6} in RSV A2 P_2 Vero

Will infect 25cm² bottles with "Undil 18537 P_4 Vero" and dil.
" " " " with New 18537 (from Judy Beeler) dil / Undil } 0.5ml each infection
" " " " with New A2 Undil and dil.

11am did infections – adsorbed 2hrs at 37°c.

Prepared some fresh virus growth medium ABiot +, Gln +, NEAA +, FCS–heat inact +.

RSV A2 Vero P2 may not be giving as high a titer this time around for a number of reasons

1. There was no Hepes / $MgSO_4$ in the supernatant – used to stabilise the virus
2. Vero cells may be are becoming refractile to RSV (at higher passage N°.)
3. Cell monolayers were a little sparse

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Recorded by David K Clarke

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Checked $TCID_{50s}$ for 18537 p_4 Vero dil/undil and A2 p_2 Vero

It seems that there are 3 wells showing c.p.e. at 10^{-5} dil^n on 18537 p_4 Vero undil. which would translate into a titer of $10^{5.25}$. In A2 p_2 Vero - there is c.p.e out to 10^{-7} dil^n ≡ $10^{7.5}$ Will make final read-out tomorrow morning

Checked cytopathic effect on all three sets of infections - there are already some indications of c.pe on some of the monolayers - especially the one infected with A2 pp3D undil.

Ordered 2,500 units of RNasin from Promega $75.

Did final check on c.pe for today - looks as though there is already recognizable giant cell formation in most of the infections - will harvest either tomorrow morning or thurs morning

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HDA

Recorded by David K Clarke

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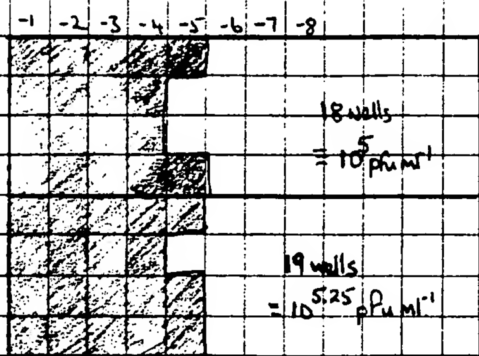
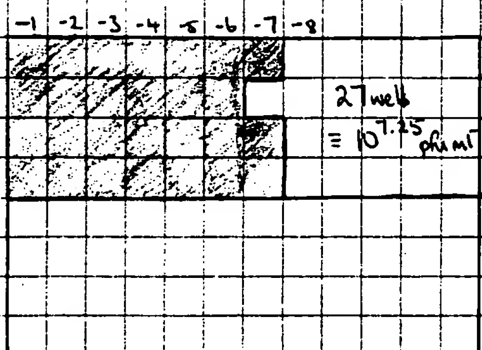
Checked $TCID_{50s}$ for Final read



18537 P_4 Vero

Dil

Undil



RSV A2 p_2 Vero

Undil.

Checked cytopathic effect on infected $25cm^2$ flasks.

① * RSV A2 (New vial from J. Beeler) – Dil – Good c.p.e at 48h p.i.
Undil – Possible interference but cells look sick. will leave a 3rd day.

* The 'grungies' observed in the 1st vial of this stock are again present, indicating J Beeler stocks are contaminated with either yeast/fungus or pen/strep resistant bacteria.

② RSV 18537 pp2 new stock of 'B virus' from J. Beeler. Not much c.p.e yet at 48h p.i. - will change medium for shock treatment to boost virus production.

③ RSV 18537 P_4 Vero Dil – cpe at both dilutions – will
Undil – leave for a 3rd day.

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6pm Checked c.p.e again. 2A infected cells are showing gross c.p.e - greatest at 'dilute' - equivalent to 50µl of undilute stock
Will use 150-200µl of stock (J. Beeler) + 300µl MEM containing Fungizone to infect a 75cm² bottle.
Need to add 1ml Fungizone to 500ml MEM or 0.1ml/50ml or 50µl/25ml.

Will infect 1 x 75cm² bottle with 0.5ml of ATCC RSV A2.

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Checked cpe on infected cell monolayers

A2 - P1 Vero 2nd batch Dil. Undil } Cells blown away. complete cpe.

1st batch 1853? P5 Vero Dil Undil } Cells blown away - complete cpe in Undil - little less cpe in dil.

Harvested these four flasks separately and froze down cells at -90°C

Will infect 1x 75cm² Flask with 200µl of A2 (+300µl MEM) - from [redacted] Judy Beeler PP3D Hep 4 in presence of Fungizone (50µl/25ml)

Will infect 1x75cm² flask with 0.5ml of ATCC A2 virus.

T/A cloning kit from Invitrogen received.

Absorbed virus for ≈2hrs at 37°C

Ordered cells for next week.

5th day harvest!

Carried out $TCID_{50}$ on A2 P1 Vero 2nd Batch (also contains "gauagies") as a matter of interest. Dil + Undil.

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Witnessed & Understood by me, Holly Abrams

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Invented by

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Recorded by David K Clarke

From Page No.

Checked $TCID_{50}$ of A2 P_1 Vero 2nd batch (contaminated). Not yet any significant c.p.e.

4th day p.i. Checked 18537 p_1 Vero (New) - Not showing dramatic c.p.e. Will harvest until today and run a $TCID_{50}$ on Mon.

Checked A2 infected cells - J. Beeler 3rd batch + Fungazone
- ATCC virus
Both monolayers are showing significant c.p.e. at 24h p.i. Will harvest either on Sat or Sun.

Dr Kevin Moore (ILID) Gave a seminar at lunch time

Infected 2 × $25cm^2$ flasks with 18537 P_5 Vero (300µl) - one flask with virus from Dil. P_5 Vero - one flask with virus from undil P_5 Vero - Adsorbed ≈ 2 hrs (used undil inoculum in both cases)

Did $TCID_{50}$ on 18537 P_5 Vero Dil.
Undil.

Harvested 18537 p_1 Vero (New batch) dil undil even though cytopathology was not very significant.
Froze and stored in Upright freezer - 2nd shelf from the bottom.

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Checked c.p.e on RSV A2 infected cells. p_1 Vero

ATCC. } c.p.e v. extensive on both.
J. Beeler. }

Could still see contaminating bug in J. Beelers prep.
∴ assume it is $Pen^R/Strep^R$ + $Fung^R$. Will filter sterilise virus harvest and build titers back up if there is a loss.

Harvested both Flasks of virus. - Froze at -90°c. (bottom tray)

Checked c.p.e on $TCID_{50}$ J. Beelers RSV A2 p_1 vero Dil/Untitl; c.p.e visible out to 10^{-5}.

No appreciable c.p.e. yet on RSV p_6 Vero.

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Checked TCID$_{50}$ of ~~(18537)~~ A2 p_1 Vero, 2^n batch — Undil gave highest titer ≈ $10^{6.5}$ — will make final reading tomorrow.

Checked c.p.e. on 18537 p_5 Vero Dil } infected 25cm^2 bottles — c.p.e. is Undil } not as extensive as I would have anticipated — it appears the cells are becoming refractile to virus growth.

Will wait for newer cells to start cell cloning work (next mon.) meantime will harvest 18537 p6 Veros later today for comparison with similar 3 day infection with newer cells.

Infected 2 x 75cm^2 Flasks with { A2 P_1 Vero Undil ATCC
A2 P_1 Vero Undil J.Beeler*

Adsorbed ≈ 2hrs at 37°C

*Slightly lighter cell monolayer.

Set up TCID$_{50}$ on A2 p1 Vero ATCC
J.Beeler } ran up til after 10^{-5} dil^n — shouldn't be a problem though.

(3 days p.i.) Harvested virus from 18537 p_6 Vero Dil/undil — froze down for comparison with p_6 Vero from younger cells.

Checked TCID$_{50s}$ again — still looks as though there is a reduction in the number of viruses produced — possibly due

May go back to 18537 p2 Vero when the new cells are ready in order to try and boost titers even more (from the max of $10^{5.5}$ observed for P_2 Vero 18537

Harvesting a little early — rather than waiting for total destruction of cells may also be important

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Witnessed & Understood by me, Holly Abrams | Date | Invented by | Date

Recorded by David K Clarke

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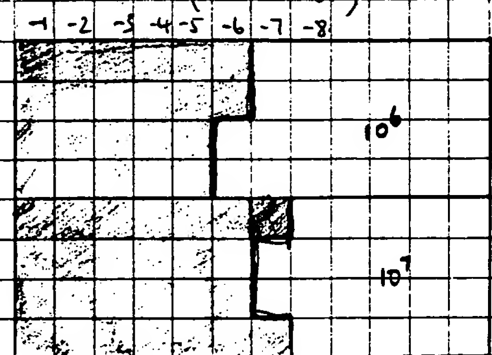
Analysis of cDNA (S5.) by Alk. gel elect. is in sec'n 6.2 Mania to.

Checked 75cm² Flasks infected with RSV A2 p_1 Vero ATCC — shows more advanced c.p.e.
J. Beeler — not much c.p.e visible.

Overall, the level of c.pe observed was lower than expected indicating that virus may be restricted somewhat by the older cells.

Checked TCID50s A2 p_1 Vero 2nd batch — titer appears to be $10^{6.75}$ on undil (lower in dil)



Dil.

RSV A2 p_1 Vero 2nd batch (J Beeler)

undil

Checked TCID50 of 18537 p5 Vero — There is c.p.e out to 10^{-4} in some cases — however there appears to be some drop in titer — again possibly reflecting refractility of Vero cells. (Kim says they are ≈14 passages out from frozen — should change at 10-12 passages)

I am looking forward to comparing New/old cell titers with 18537 p5 Vero.

Important Notes

① Use young cells [redacted] ≥10 passages from frozen.
② Adsorb virus in small volume for 2-3hrs with high M.O.I. as possible.
③ Time of harvest may be important; e.g. too late may result in drop in titer due to virus ½ life

May go back to 18537 p_2 Vero (which had $10^{5.5}$ titer) in order to try and boost titers (next week).

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Witnessed & Understood by me, Holly Abrams

Date

Invented by

Date

Recorded by David K Clarke.

Project No.

TITLE Book No.

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5-30pm checked c.p.e on RSV A2 P_2 Vero JBealer ATCC ; again. Cpe appears to be

– progressing reasonably well – will check again tomorrow morning.

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George Kible		Recorded by David K Clarke	

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Checked A2 infected cells – cpe is well advanced in ATCC infected cells (p_2 Vero) and in J.Beeler infected cells – may harvest this evening or tomorrow morning. Will filter sterilise J.Beeler stock and include tetracycline in the growth medium.

Did final reading on RSV A2 p_1 Vero 2nd Batch J.Beeler – Results indicate a titer of Dil/Undil (see Back page) $\frac{10^7 \text{ Undil}}{10^6 \text{ dil}}$

Checked TCID$_{50}$ of 18537 p_5 Vero — Tomorrow will make a final reading. It appears there is cpe out to 10^{-4} in Dil (21 wells would be a titer of $10^{4.75}$) – down on previous titers. It looks as though the cells are refractile (more so) than during earlier passage N°.

2 days p.i. There is not much information available on TCID$_{50}$ for A2 p_1 Vero J.Beeler 3rd Batch + ATCC Virus.

Carried out TCID$_{50}$ on 18537 p_1 Vero 2nd Batch – don't expect much of a titer.

Harvested A2 p_2 Vero J.Beeler – Extensive c.p.e. – but heavily contaminated.

ATCC
↓
Advanced cpe. Decided to harvest rather than leave o/n.
↓
froze at -70°C upright freezer.

Different ← from J.Beeler c.p.e which was more fusogenic.

J.Beeler ↓ Filter sterilised after harvest. Aliquotted and froze in upright freezer at -70°C.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

From Page No.

Dil Final check on $TCID_{50}$ on $18537p_5$ Vero 1st Batch

-1 -2 -3 -4 -5 -6 -7 -8 -9

$10^{4.25}$ Undil

RSV $18537p_5$ Vero

$17 = 10^{4.75}$ Dil

Overall impression that cell refractivity is acting on virus growth.

Checked cpe on $TCID_{50}$ for RSV A2 P_1 Vero - ATCC - J.Beeler } cpe in both out to 10^{-4} v. different in nature - fusogenic in J.Beelers - Giant cell formation with ATCC strain.

Checked $TCID_{50}$ for $18537p_1$ Vero 2nd batch - No sign of any cpe yet.

Ordered cells for next week

For Mon 2x96 well plates; One to compare $18537p_5$ Vero titers
One to check A2 P_3 Vero stocks - ATCC / J.Beeler
1 x 25cm² Flask To do vero cell cloning
3 x 75cm² Flasks - One to amplify $18537 p_2$ Vero (highest titer so far for B subtype) in newer cells to try and boost titer
Two for A2 growth (Either Dil/undil of one stock or undil of both stocks)

For Thur 4 x 75cm² Flasks to prepare working stock of either ATCC or J. Beeler A2.
2 x 96 well plates - one to titer A2
One to check titer on $18537 P_2 \rightarrow P_3$ Vero titer

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From Page No. 35

Set up $TCID_{50}$ on RSV A2 P_2 Vero – got slight mix up in Dilutions but will be able to differentiate ATCC and J. Beeler virus based on c.p.e unless removal of bacterial contaminant affects c.p.e

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Witnessed & Understood by me,	Date	Invented by	Date
[illegible]		Recorded by Daniel K Clarke	

Jodie and Paulette Leave Today

TITLE: Project No.

Book No.

From Page No.

Checked TCID$_{50}$ Day 4 for A2 p_1 Vero ATCC – c.p.e to 10^{-4}
J. Beeler – c.p.e. to $10^{-5/-6}$

TCID$_{50}$ for 18537 P_1 Vero 2nd batch – No c.p.e yet visible – virus grew in v. sickly way, so perhaps there were v. few virus produced.

Infected 75cm^2 flasks – One with 0.5ml of ATCC A2 p_2 Vero
one with 0.5ml of J. Beeler A2 P_2 Vero. – Filter sterilised.
Adsorbed ≈ 2-3hrs. Added 10ml of MEM containing Tetracycline ($\frac{1}{250}$th of stock)

No c.p.e yet visible on P_2 Vero A2 TCID$_{50}$.

2-15pm infected 2 x 25cm^2 flasks with 18537 P_4 Vero Undil. Made a 10^{-1} dil^n and infected one flask with 0.5ml 10^{-1} dil^n and one flask with 0.5ml undil. Used new Vero cells to try and get a comparison with older Veros (with respect to titer).

Checked CO_2 Incubators temp./CO_2 conc. – changed H_2O.
↓
~~Adj~~ Measure on Mon after CO_2/Temp equilibrates.

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Serge Kobbe [signature]		Recorded by David K Clarke [signature]	

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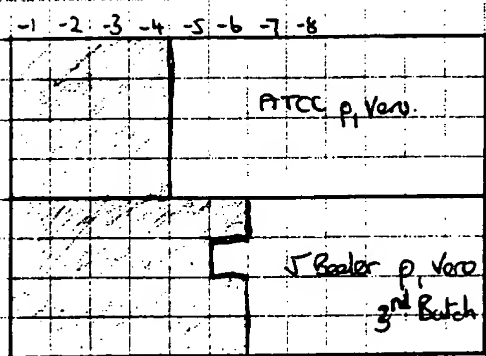
2pm. Checked Infected cells + $TCID_{50}$s

A2 (ATCC) infected cells (p_3 Vero) until were wiped out 24hpi. Harvested these cells/virus and stored at -80°C in Upright Freezer.
A2 (J Beeler - filter sterilized) (p_3 Vero) until - No c.p.e at 24hpi - Either all the virus stuck to the filter, or all the c.p.e seen previously was bacterial induced. There was no sign of the bacterial contaminant.
I will try spinning some previous P_2 Vero A2 J.Beeler in the microfuge 2 x 10mins - and infect in the presence of tetracycline.

Checked $TCID_{50}$ for P_1 Vero ATCC/J Beeler A2



$\approx 10^{4.5}$

Contains contaminant $10^{6.25}$ ∴ invalid

Checked $TCID_{50}$ p_2 Vero ATCC/J Beeler Until

ATCC virus is showing improved titer - will make a final reading on mon or tues.

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Recorded by

John's Lab Fax No.
(619) 534-7108.

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Book No.

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Checked 18537 p5 Vero infected cells (New cells) The c.p.e. is v. extensive at Undil - not so extensive at dil.
Harvested both - froze at -80°C

Carried out TCID$_{50}$ on 18537 p5 Vero Dil/Undil 'New cells' to get a comparison with the titer from older cells

Also did a TCID$_{50}$ for A2 ATCC P$_3$ Vero

Checked TCID$_{50}$ for A2 P$_2$ Vero ATCC - c.p.e. out to 10^{-5} dil^n ≡ $10^{5.5}$ titer ml^{-1}
" " for 18537 2nd batch (J Beeler) c.p.e. out to 10^{-3} dil^n = 10^3 titer.
Will make some final readings tomorrow.

Infected 1 x 75cm^2 Flask with A2 P$_2$ Vero (1st batch) Undil after spinning ≈ 1.2ml for 10mins → 1ml spun for a further 5mins → used 0.6ml to infect a monolayer (cells were not v. confluent ≈ 50%)

Infected 1x 75cm^2 Flask with A2 ATCC P$_3$ Vero Undil (used 0.6ml).

Infected 1 x 75cm^2 Flask with 18537 P$_2$ Vero (went back to older stocks) - which had a high titer ($10^{5.5}$) ≈ 0.5ml inoculum

Ordered 500u of Life Sciences Avian Reverse Transcriptase XL.

Kim set up Vero cell cloning for me 100 cells / 10ml MEM = 1cell/100µl

Completed measurements of CO_2 etc on incubators in Resp Virus lab.

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Serge Keble		Recorded by David K Clarke	

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Checked TCID$_{50}$s on RSV A2 ATCC/J Beeler p_2 Vero
RSV 18537 p_1 Vero 2nd batch

-1	-2	-3	-4	-5	-6	-7	-8		
					$10^{3.25}$			Dil	RSV 18537 p_1 Vero 2nd batch
					$10^{2.75}$			Undil	

Perhaps there are a lot of DIs
Most probably will not proceed with this stock of 18537 as I already have quite a good growing stock

-1	-2	-3	-4	-5	-6	-7	-8		
					$10^{5.5}$				ATCC undil. p_2 Vero
									J. Beeler Undil (filter sterilised)

Checked Infected cells. Only ATCC infected (now p_2 Vero) - Undil - cells are showing c.p.e., they should be ready to harvest tomorrow

Took a look at Vero clones (single cell stage) - There are clearly some wells containing only one cell - in some instances there were more than one cell and in one case one cell was undergoing cell division.

Checked TCID$_{50}$s for ATCC p_3 Vero - 24h.p.i. - too early for meaningful assessment

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Witnessed & Understood by me,	Date	Invented by	Date
Serge Kibble (signature)		Recorded by David K. Clarke (signature)	

Dr. Jorge Gomez. (415) 725-7496 (Lab)
Project No.______
TITLE ____ (510) 796 6277 home. Book No.______

From Page No.__

Checked infected cells - ATCC p4 virus are showing extensive c.p.e. - will harvest them this afternoon - no cpe yet on J Beeler infected cells (after super was spun for 15mins in microfuge). All virus appear to be cell associated.

C.p.e. has begun on 18537 p2 Vero-infected cells - but it will probably be at least another day before they are ready for harvest.

Reverse Transcriptase arrived - stored at -80°C in Wright Freezer

Harvested ATCC Virus P_4 Vero - aliquotted and stored at -80°C. There may be a slight reduction in c.p.e. relative to the ATCC P_3 Vero - possibly due to DI particles - however this

On looking over previous papers on RSV sequencing (genomic RNA) a number of things can be concluded.

1. Can expect ≈ 20 μg of genomic RNA from ≈ 20 roller bottles = ≈ 80 TC 175 cm² bottles.

Need RNase A. or/and Micrococcal nuclease

2. Can expect a high level of cellular RNA/DNA contamination - may want to add RNase A, DNAse to super prior to spinning out virus either while still in MEM or prior to loading on sucrose gradient

Need Formaldehyde deionised Formamide. Filter Clone containing RSV cDNA.

3. Should probably analyse a portion of each genomic prep on either a 3% Acrylamide gel (see D Stec) and silver stain, or on a 1% Mops/Formaldehyde gel and blot + probe

Order dNTPs.

4. Analyse ~~cDNA~~ synthesis on hydroxide gel with markers. (need ^{32}P or ^{35}S) Use equiv. of 1-2 μg so have RNA remaining for cloning

5. Set up PCR conditions carefully - taking all available knowledge into account.

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[signature]		Recorded by David K Clarke.	

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Calculation of virus inoculum size for infection of Roller bottles.

Surface area calculations indicate that 11.33x inoculum will be required for a roller bottle vs a $75cm^2$ bottle.

Total inoculation vol. will be ≈ 5ml – can probably dilute working stock 1/10 for infection i.e. 0.5 + 4.5ml → 1 roller bottle. However a smaller inoculation vol. may be possible due to the rolling nature of adsorption.

Ordered: Proteinase K, DNase I, Micrococcal Nuclease } Boehringer.

Did final check on 18537 p_2 Vero infected cells – cpe has progressed but will not be ready for harvest until tomorrow afternoon.

Need to check that we have the facility to handle 20 roller bottles worth of supernatants.

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Recorded by

From Page No.

Checked c.p.e. on 18537 p_2 Vero infected 'New cells' - 3 days p.i.
The c.p.e was very extensive so decided to harvest - Froze down as 18537 p3 Vero New cells.
Will do a $TCID_{50}$ on this stock this afternoon along with ATCC p_4 Vero stock (harvested yesterday).

Checked $TCID_{50}s$ - will make final readings in 1-2 days time.

Checked Vero clones - marked off those containing one clone only. Looks as though there will be about 40 Vero clones total.

Infected 4 x 75cm² flasks with ATCC p_4 Vero 0.5ml x2 (10^{-1} dil^n)
0.5ml x2 (undil)

Did $TCID_{50}s$ for ATCC p_4 Vero and 18537 p_3 Vero 'New cells'

Interviewed Peter Brown.

Ordered cells for next week.

MON - 4 x 75cm² bottles (for more ATCC virus?)
2 x 25cm² bottles (for more 18537 virus?)

Thur 2 x 96 well plates

Fri 20 Roller bottles

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Witnessed & Understood by me, Serge W Kolbe | Date | Invented by | Date

Recorded by David K Clarke

Zellerbach – for Ice cream Cartons.

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Checked ATCC TCID$_{50}$ P$_5$ Vero – In some cases c.p.e. was visible out to 10^{-6} indicating a titer close to 10^{6}.

Checked 18537 TCID$_{50}$ P$_5$ Vero (for comparison with older cell titer) – Appears to be cpe out to 10^{-5} in some cases – indicating that the newer cells make a real difference in growth capabilities for RSV.

Checked ATCC infected cells (P$_5$ Vero di/Undil) – No significant c.p.e. yet; however it is only ≈ 20h.p.i. – will check again late this afternoon. May have to harvest tomorrow.

Checked Vero cell clones – they are growing quite well; will refeed on Mon. with 100–150μl of fresh growth medium.

Fridge – Freezer arrived from Sears – installed in My/Ying's lab.

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Witnessed & Understood by me,	Date	Invented by	Date
Serge Kalble		Recorded by David K. Clarke	

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From Page No.

Checked c.p.e. on ATCC infected P_5 Veros. Not very extensive c.p.e. at 48h.p.i. – something is wrong! Either the adsorption period was insufficient or there has been significant build up of DI particles (latter is most likely since this virus has been passaged undil. for 5 series). Will go back to P_2 or P_3 Vero to prepare working stock.

MON

3hr adsorption { Will infect each 75cm² with 0.5ml of ATCC P_2 Vero → working stock.
Will infect each 25cm² with 0.2ml of ATCC P_3 Vero 10^{-1}/undil.

If c.p.e. look good at 10^{-1} dil^n of P_3 Vero – will use that dilut^n to infect roller bottles

Checked $TCID_{50s}$ for ATCC P_3 Vero and 18537 P_5 Vero (New cell) – Will make a final reading on Monday, but it looks as though the ~~titer~~ titers will be $10^{6.25}$ and $10^{5.25}$ respectively. This represents a ½ log improvement for 18537 – simply by using newer cells; it will be interesting to see what the 18537 P_3 Vero (New cell) titers will be.

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Recorded by David K Clarke

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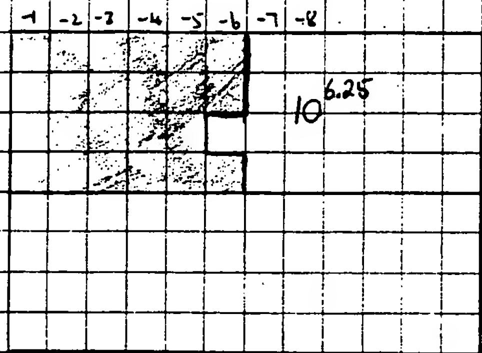
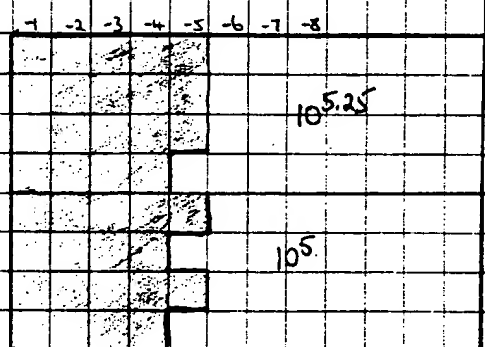
Made final $TCID_{50}$ readings



$10^{6.25}$

ATCC Virus
p3 Vero Undil.

This is the highest titer for ATCC virus so far - probably helped by the 'New cells'



$10^{5.25}$

10^{5}

18537 Virus
P_3 Vero ('New cells')

The dilute virus gave slightly higher titers this time and the previous time (Oct 7th). The Newer cells gave ≈ ½ log higher titer than the older cells used in 7th Oct

Checked $TCID_{50}$ for ATCC P_4 Vero Undil → appears that titer is not as high as P_3 Vero ATCC indicating the presence of DI particles. Probably should have used diluted P_3 Vero for infection

Will make a final reading on 18537 p3 Vero "New cells" on Wed.

10-30am Infected 4 x 75cm² Flasks with ½ diluted (0.5ml) P_2 Vero ATCC.
Infected 2 x 25cm Flasks with ATCC p3 Vero 10^{-1} dil and Undil (200µl for each infectⁿ)
adsorbed 3hrs (added 1ml Hepes buffer to MEM ≈ 200ml to stabilise pH.)
Checked Vero cell clones - many cells appear to have died off - perhaps I had them out of the incubator too long (pH or temp affected). Will feed with some fresh growth medium this afternoon.

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Recorded by David K. Clarke

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Aspirated off some of the medium from the cloned Vero cells - replaced it with 150µl of fresh growth medium.

Staining RNA after transfer to nylon membrane/nitrocellulose

① Soak dried filter in 5% Acetic acid for 15mins at room temp.

② Transfer the filter to a soln of 0.5M NaOAc (pH 5.2) and 0.04% methylene blue for 5-10mins at r.temp.

③ Rinse the filter in H_2O for 5-10 mins. RNAs used as M.W. standards should appear as sharp bands.

* Herrin and Schmidt (1988). Rapid, reversible staining of N. blots prior to hybridisation. Biotechniques 6: 196.

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From Page No.

Checked $TCID_{50s}$ - ATCC p_4 Vero. - c.p.e only to 10^{-4} or so indicating heavy buildup of DI particles, reducing titer. Also 18577 p_3 Vero (New cells) is indicating a titer of $\approx 10^5$ - a little lower than the original $10^{5.5}$ p_2 Vero. Will make final readings of these $TCID_{50s}$ tomorrow.

Checked P_2 Vero and P_3 Vero (ATCC) infected cells - just beginning to show c.p.e. - will check again late this afternoon.

Checked Vero Clones. - perhaps only $\simeq$ 5-10 clones will survive - will need to step up efforts in future.

prepared a large batch of solutions in preparation for viral RNA production.

1M Tris pH7.5 - Autoclave
1M NaAcetate pH 4.5. - Autoclave.
20% SDS (Not autoclaved)
Glycerol - autoclave
1M $MgCl_2$ autoclave
1M $CaCl_2$ autoclave
0.5M EDTA pH8.0. autoclave
X10 TE buffer pH7.6
X10 NTE buffer pH7.6

C.P.e. progressing well on ATCC infected cells. $P_2 \rightarrow P_3$ Vero at ½ dilution and $P_{3\,Vero} \rightarrow P_{4\,Vero}$ dilut^n 10^{-1}/undil. Will probably be able to harvest tomorrow this afternoon

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Recorded by

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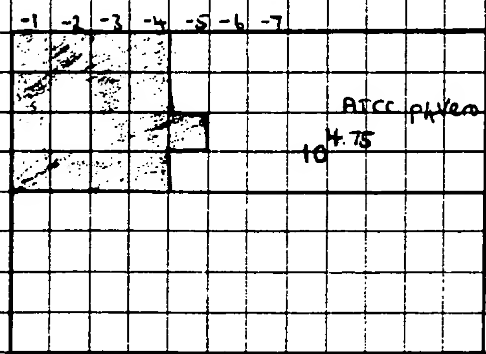
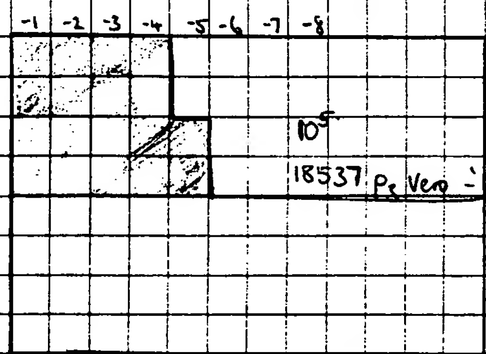
Checked TCID$_{50}$s - final reading - titers are low



DI build up may be a problem



18537 P_3 Vero - 'New cells' - Perhaps should have infected with 10^{-1} dil^n (DI build up may be a problem once the titer goes over 10^5

Checked Vero cell clones - there appears to be ≈ 9-10 clones growing slowly
Checked c.p.e. on ATCC infected cells - they are progressing very well - will harvest ≈ 4pm

prepared Micrococcal nuclease stock at 1mg ml^{-1} - stored frozen (in deionised H_2O)
prepared DNase I 2.5mg ml^{-1} in 5mM NaAc 1mM $CaCl_2$ 50% glycerol - stored -20°C
prepared Proteinase K at 10mg ml^{-1} in 10mM $CaCl_2$

By 5pm the c.p.e. on P_3 Vero and P_4 Vero ATCC infected cells was very extensive - decided to harvest them all even though 10^{-1} P_4 Vero was not as extensive in c.p.e. (stored all in "Working Stock box" at -80°C).

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Witnessed & Understood by me,	Date	Invented by	Date
Serge Kibble		Recorded by David K Clarke.	

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Carried out $TCID_{50s}$ for P_4 Vero (ATCC) Dil/until - as a test for roller bottle infection with P_3 stock.

Also did $TCID_{50}$ for ATCC P_3 Vero - To be used as working stock for infection of Roller Bottles.

Moved Nucleases + Proteinase-K to 'General Freezer.'

* Vero Cloning

Kim recommended using 1ml of growth medium for 24 well plates and 2ml for 6 well plates - Rinse briefly with some PBS - Trypsinize by pipetting up and down with a yellow tip.

Lunch with Frank.

Went over protocols with Kathy - centrifuges, tubes, volumes, speeds etc.

Checked cloned Vero cells; a few more clones are showing signs of life.
Where cells are growing they have a very

Checked Temps/CO_2/H_2O in Flu/RSV T.C. Room.

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Witnessed & Understood by me, Sego Kible | Date | Invented by | Date

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From Page No.

9am Flu/RSV group scientific meeting

12-1pm Infected 20 Roller bottles with RSV A2 ATCC P_3 Vero – Diluted 1/10 in MEM
Used 10ml of this dilution to infect each roller bottle.
Absorbed virus for x 3hrs at 37°c in the roller cabinet
Then added 45ml of MEM → 37°c for 48hrs – 72hrs

George provided me with a bottle of Ultrapure Formamide – stored in freezer in My/Ying's Lab. – will deionise before use and refreeze at -80°c. Need to order mixed bed resin

Proportions for MOPS Gel.
Per 50ml
5ml x10 MOPS buffer
36ml H_2O Check vs. Ying's protocol.
9ml Formaldehyde
Melt agar in H_2O, ~~cool~~, then add x10 MOPS and Formaldehyde – cool further and pour.

Checked $TCID_{50}$s on ATCC P_3 Vero working stock and P_4 Veros 10^{-1}/ undil.
There are the beginnings of c.p.e. – will check again on Monday.

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Recorded by David K Clarke

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From Page No.

Checked infected Roller bottles. There has been considerable cpe caused by the virus (RSV A2 ATCC P_3 Vero diluted 10^{-1} – 10ml/Flask). Many dead cells are floating along with clumped dead cells and syncytia. Some syncytia are also visible on the remaining cells. There are sufficient remaining cells to warrant leaving until tomorrow. Hopefully by then the majority of cells will have been infected and will be free floating.

Also checked $TCID_{50}$s for ATCC P_3 Vero and ATCC P_4 Vero dil/undil. They are showing good cpe out to 10^{-4} – will make a final reading on 27^{th} (wed).

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Witnessed & Understood by me, Serge Kble | Date | Invented by | Date

Recorded by David K Clarke

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Checked $TCID_{50}$ - looks as though there is c.p.e. out to 10^{-5} with ATCC p2 Vero (working stock) but only to 10^{-4} with P_4 Vero 10^{-1}/undil. Will make final readings on Wed.

Assuming $10^{5.5}$ ml^{-1} for ATCC p_3 Vero may need to dilute by 10^{-2} and have 5×10^3 pfu ml^{-1} (This should eliminate majority of DIs) then put 200µl on a 6well plate x5. Also will try sonication in a controlled experiment.

Checked Vero clones - they are doing fine and showing signs of growth - will change medium again on Thurs or Fri.

Checked cells for passage (Veros, left for me by Kim) - Will split tomorrow into $2\times25cm^2$ flasks for use on wed, a 96 well plate for thurs or fri + a TC150 for fri to be passaged for the following week.

Harvested virus from 20 Roller bottles - Spun at 3K rpm for ≃10mins for Cond spin then freeze-thawed pellets each in 5ml MEM, repelleted for 5mins at 2Krpm pooled the supers and then put on O/N spin. Retained 1.5ml of super for $TCID_{50}$.

Ordered mixed-bed resin from BioRad 500g $110.

Set up $TCID_{50}$ on Roller bottle harvest

Put on O/N centrifuge spin 10K RPM 4°c in the '14' Rotor (Beckman JA14) Rotor.

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Witnessed & Understood by me, Sez Kble | Date | Invented by | Date

Recorded by David-K Clarke.

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Took off virus-pelleting spin – there were substantial pellets visible in each centrifuge bottle – Decanted supernatants, drained and added 1.0ml of nuclease compatible buffer onto each pellet. (Buffer contained 50mM Tris pH7.5, 10mM $MgCl_2$, 5mM $CaCl_2$
50µl Tris pH7.5 1M, 100µl $MgCl_2$ 1M, 50µl $CaCl_2$ 1M + 9.35ml H_2O)

Left pellets to resuspend o/n on ice in the cold room.

Did some cell culture – Trypsinized Vero cells from TC 150cm² into 1ml of Tryp-EDTA. Used 200µl to set up a fresh TC 150cm² flask. Also used 200µl → 8ml MEM for 2 x 25cm² flasks. Diluted 100µl into 10ml MEM, then diluted 2ml of that into 18ml of MEM and plated out 200µl/well of a 96 well plate. Cells look a little dilute, but they should be ready by mon at the latest.

Prepared a batch of 20 x SSC – 3 Litres – stored in Mol. Biol. Lab.

Checked $TCID_{50}$s – will make some final readings tomorrow

To Page No.

Witnessed & Understood by me, Sergei Kibble

Date

Invented by

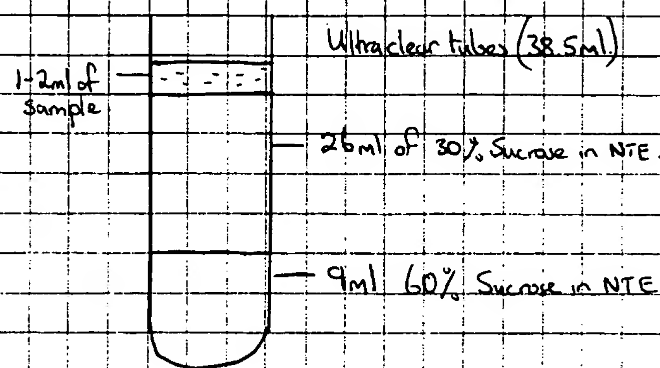
Date

Recorded by David K Clarke.

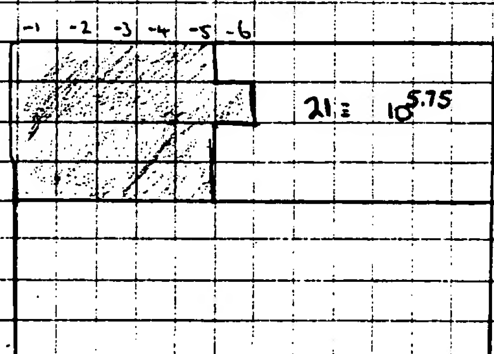
Checked Virus/cell debris pellets – resuspended them by gentle pipetting (1ml tip) – Transferred to eppendorf tubes x 5. Treated one tube (A) with DNase I ≈ 1ug/ml (50µl of stock)
Micrococcal Nucl. ≈ 1ug/ml (100µl of stock actually used 50µl)
RNase A. (10µl of 10mg/ml⁻¹ stock)

9-45 – 10-45am

Prepared Sucrose gradients for banding of Virus



Centrifuged at 27K for 45mins at 4°C SW 28 rotor



$21 = 10^{5.75}$ ATCC p_3 Vero (Used as ~~seed for~~ working stock)

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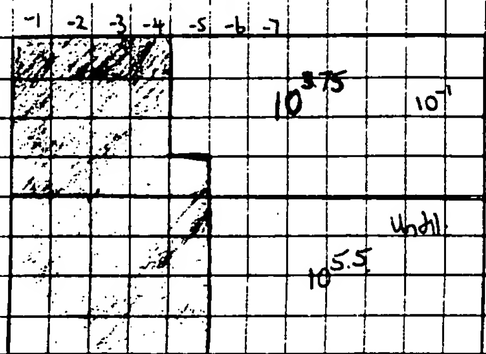

Checked cell monolayers which I set up yesterday.

The 25cm² Flasks will be ready tomorrow.
the TC 150 will be ready for passage on Fri - the 96 well plates should also be ready by Fri.

After Nuclease treatment of Sample A diluted in NTE and repelleted along with the untreated B sample (NB let a lot of cell debris settle out of both samples - only pelleted the material above the settled debris.

After running sucrose gradient, saw a large milky band at the interface of the 30% sucrose and 60% sucrose, along with much cellular debris. Harvested off the bands - mixed with STE and repelleted along with A + B above in the SW 28 (38.5ml buckets)

3-45 spin on. ~~the 40mins at 27K 4°C.~~

(It does not seem at the moment that the sucrose gradient has been of much use in the purification scheme, but we must wait and see.)

Decanted supers from pellets, and resuspended (O/N on ice) A and B in STE x1 - resuspended C and D in nuclease compatible buffer. Used 1ml for resuspension.

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Witnessed & Understood by me, Serge Koble

Date

Invented by

Date

Recorded by David K Clarke.

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Resuspended "virus pellets" in either S.T.E or nuclease compatible buffer
A + B in STE   C + D in Nuclease compatible buffer. Added 50µl of Stock DNase I + 50µl of stock Micrococcal nuclease + 1µl RNase A — mixed and incubated at 37°C for 1hr (9am – 10am)
(Inhibited Micrococcal nuclease with EDTA)
Following Nuclease treatment will set up proteinase K reaction in x1 NTE, 0.2% SDS and 1mg ml^{-1} proteinase K. – 1hr at 37°C

Checked Vero clones – not a large amount of growth – will remove some old medium and add some fresh medium this afternoon.

Preparing Proteinase K in $CaCl_2$ may have been a mistake since the $CaCl_2$ may supply enough Ca^{2+} for micrococcal nuclease – although there should be enough EDTA to take care of it.

Infected 2 x 25cm^2 flasks, each with 200µl of ATCC p_3 Vero + adsorbed for 2-3hrs

Replaced some of the growth medium on the Vero clones with fresh growth medium

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Witnessed & Understood by me,	Date	Invented by	Date
Serge Kobel		Recorded by David K Clarke	

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Spun down 1ml from each of genomic RNA preps - dried down pellets (looked a little too large and white to be RNA - they are probably DNA)

Prepared 10M NaOH, 1M NaCl, 1% methylene blue, 1M NaAc pH5.2 in preparation for Northern blot work.

Resuspended my pellets in 10µl x1 MOPS gel loading buffer, 10µl Ultra Pure Formamide and heated to 60°C for 5-10mins. Then added 5µl of Ficol/BPB and loaded gel.

L.H.S

/5µg Plasmid DNA / 0.2µl (1µg ladder) / 2µl (2µg) ladder / A / B / C / D /

Run gel ~~for~~ at 80mA until BPB was at the bottom of the gel

Need to check infections (from yesterday), 96 well plates; set up TC150 for Mon/Tues.

Infection is proceeding well, will harvest tomorrow afternoon - sonicate +/-

Set up a TC150 for monday using 200µl out of (1000µl of trypsinised cells)

Proceeded with N.Blot after the BPB had run to the end of the gel. - Soaked in 3 changes of H_2O (each for 5mins - to remove Form.) then soaked in 50mM NaOH 10mM NaCl for 15mins then for ≈1hr in 100mM Tris pH7.5 (this could be shortened), then for 30mins in 20x SSC - Then set up blot.

Will dismantle tomorrow and U.V. cross-link on RNA. Will wait until Monday to stain the filter.

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		Recorded by	

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From Page No.

Dismantled N Blot (from yesterday) - allowed filter to air dry - it was still damp, and then U.V. cross-linked XR using the settings George showed me. Stored in my Lab Drawer at R.Temp

Checked infected Veros - c.p.e was v. extensive - harvested both $25cm^2$ Flasks.

Sonicated one harvest (3x 2secs at setting Frances uses) - sonication was very powerful and frothed the medium and disintegrated the cell debris.
Freeze-thawed the other harvest - then carried out a $TCID_{50}$ to compare pfu from each harvest technique.

Tidied TC room and restacked (final week of responsibility!)

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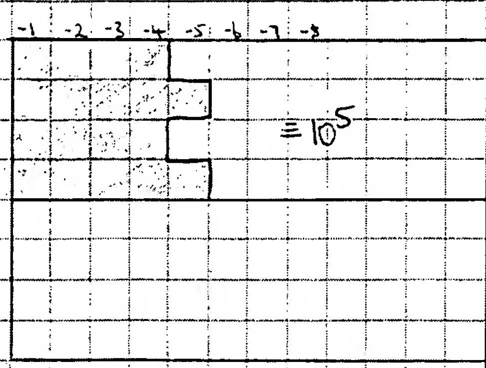
Serge Kobel		Recorded by David K Clarke	

From Page No.___

Did final Read on $TCID_{50}$ for Virus harvest from 20 Roller bottles



RSV A2 ATCC P_4 Vero (Roller bottle harvest)

It's possible that there were many more particles produced, but they lost infectivity

Checked Cloned Vero cells. There are at least 3-4 Vero clones which are growing quite well, will check again on Wed and will refeed and/or subclone cells into 24 well plates

Carried out staining of N. Blot filter

15 mins in 5% Acetic Acid
10 mins in 0.04% Methylene Blue
2-3 rinses in H_2O

RNA MARKERS
D C B A 4d 5d P_5

[redacted]

The larger fragments on the RNA marker lanes did not transfer efficiently to the filter, which may explain the absence of any visible genomic RNA. There should be ≈ 250ng /band in the marker lane

Although this did not look encouraging, decided to proceed with Oligo synthesis for cDNA/PCR cloning

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Witnessed & Understood by me, Sage Kohl | Date | Invented by | Date

Recorded by David K Clarke

From Page No. 60

Passaged Vero cells (200µl from 1ml of trypsinized cells) into a fresh TC150

Decided to order 2 oligos for cDNA synthesis/PCR - located in the L-gene - at position ≈12245 (1) and position ≈12775 (2).

(1) - Primer for cDNA synthesis
5'-GTTTAACACGTGGTGAG-3' ⊕ sense will be 1st primer - i.e. for cDNA synthesis.
Called - PmlI -> HphI

50°C m.p. for both primers ~~both~~ 17mers + 18mer

(2) 5'-ATCAACATCACCTGTGAA-3' ⊖ sense - will be back primer.
Called - HphI -> PmlI.

Received Oligos 11-3-93.

✱ NB paper on 'N' gene sequence of BRSV Virology 180, p453-456 (1991)
Samal et al.

Need to check Oxford RSV paper (at home).

Dave gave me a print-out of the RSV-CAT constructs.

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From Page No.

Checked $TCID_{50}$ on Sonicated/freeze thawed comparison At the moment it looks as though both and giving the same titer ($x\ 10^5$ pfu ml^{-1}) - So much for sonication.

Contacted J. Beeler (on Maternity leave) for RSV Subgroup B Mass/9320/77 (She will be back in early Dec.

Checked references on preparation of nucleocapsid for PIV 3 and RSV - they are essentially the same as the procedure used in John's lab - need to check in methods notebook.

Ordered 2nd oligo for cDNA/PCR RxN (called it "HpaI back to PmlI").

Planned out Oligos needed for making synthetic CAT/RSV construct.

Need oligos to PCR out CAT gene from plasmid, then need (eight / six) more oligos to synthesise the
(May need to Kinase these oligos)

① Xba-CAT Oligo 5' - $\underbrace{\text{TAAC}}_{\text{4 extra nucleotides to extend Past XbaI site for cutting purposes}}\underbrace{\text{TCTAGA}}_{\text{XbaI site}}\underbrace{\text{ATGGAGAAAAAAAT}}_{\text{CAT gene}}$ 24-Mer

ordered

② Pst-CAT Oligo 5' - $\underbrace{\text{AATA}}_{\text{4nt overhang to extend past PstI cut site}}\underbrace{\text{CTGCAG}}_{\text{PstI site}}\text{TTACGCCCC}$ 19-mer

Ed. Mocarski
723-6435

New concepts - a totally or partially synthetic RSV genome

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

From Page No.__

Checked TCID$_{50}$ for Freeze-Thaw/Sonicated comparison of titer

Titers appear to be identical ≅ $10^{5.25}$ - Final reading tomorrow or Fri.

Checked Vero clones. There are 5-6 Vero clones showing fairly good growth. I will expand 2 of these clones into a 24 well plate, and will feed the remainder.

Need to Order Oligos for RSV ends (to be added to the cat gene)

① 96-mer 'Kpn to Xba RSV Plus'

5'-CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA AAC TTG CAT AAA
CCA AAA AAA TGG GGC AAA TAA GAA TTT GAT AAG TAC CAC TTA
AAT TTA ACT-3'

② 104-mer "Xba to Kpn RSV minus"

5'- CTA GAG TTA AAT TTA AGT GGT ACT TAT CAA ATT CTT ATT TGC
CCC ATT TTT TTG GTT TAT GCA AGT TTG TTG TAC GCA TTT TTT
CGC GTA ATA TGC GTC GGT AC-3'

①. 50mer "RI to Hind III: A, RSV minus"

5' - GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA TAA
TTT TTT AAA TA -3'

② 60mer "Pst I to Hind III: B, RSV minus"

5'- ACT TTT AGT GAA CTA ATC CTA AAG TTA TCA TTT TAA TCT TGG
AGG AAT AAA TTT AAA CCC - 3'

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(3) 86-mer "PstI to Hind III : C, RSV Plus"

5' TAA TCT AAT TGG TTT ATA TGT GTA TTA ACT AAA TTA CGA GAT ATT AGT TTT TGA CAC TTT TTT TCT CGT TAT AGT GAG TCG TAT TA

one correction

(4) 56-mer "Hind III to PstI : II RSV minus"

5' - AGC TTA ATA CGA CTC ACT ATA ACG AGA AAA AAA GTG TCA AAA ACT AAT ATC TCG TA - 3'

(5) 60-mer "Hind III to PstI : Y RSV minus"

5' ATT TAG TTA ATA CAC ATA TAA ACC AAT TAG ATT AGG GTT TAA ATT TAT TCC TCC AAG ATT - 3'

(6) 88-mer "Hind III to PstI X RSV minus"

5' - AAA ATG ATA ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT AAA AAA TTA TAT GAT TTT TAA TTT TTA ATA ACT ATA ATT GAA TAC TGC A - 3'

Tai An delivered Oligos for cDNA synthesis/PCR - Stored at -80°C in H_2O
a 1/10 to 1/20 dilution will give 100ng µl⁻¹ conc.

Expanded 2 Vero clones which were growing well - Trypsinized them in 50µl of Tryp-EDTA after 2 rinses with PBS - Pipetted into 100µl of MEM and transferred to a 24 well plate - then added 1ml growth medium.
Fed remainder of Vero clones with fresh growth medium.

→

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Witnessed & Understood by me, Serge Kobl[illegible] | Date | Invented by | Date

Recorded by David K Clarke

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TITLE: Book No.

From Page No. 64

Will use 1µg of CAT Flu construct for PCR of CAT gene

Conditions for CAT amplification.

1µl (1µg) of plasmid DNA - get from Dave
1.5µl of each primer (1µg µl⁻¹)
2µl 10mM dNTP
5µl x10 RXN buffer
38µl H_2O

1µl Enz after 2min 'burn' → Ice → Spin → Rtemp.

Will move from 72°C → 94°C as quickly as possible. RAMP 4
Will move slowly from 94°C to annealing temp RAMP 2/3
Will move from 46°C (annealing temp) to 72°C rxn temp at RAMP 3

1min. — 1min 94°C → 2mins
2mins 46°C → 1min
3mins 72°C

programmed PCR

Double checked Oligo sequences

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From Page No.__

9am Performance rating meeting.

Prepared 1mM Methyl mercury and 1.85M 2-Mercaptoethanol

Spun down ≈1.5ml of Genomic RNA preps A and B, 70% ethanol washed and dried - held at -10°C until ready for cDNA synthesis.

Will prepare ≈100ng µl⁻¹ working stocks of primer in H_2O.

Set up cDNA synthesis exactly according to the protocol in Lab methods book. Used ≈ 40u of reverse transcriptase

Allowed reverse transcription to proceed - then inactivated at 95°C for 5mins. At this point one of the tubes popped open and some water bath water was taken into the reaction mixture - however proceeded with both samples for PCR - overlaid with the mineral oil (beside the PCR machine). Ran program N° 28 (see Page 65 for conditions)

Need to start Hep-2 cells growing to see if higher titers can be attained in these cells vs Veros.

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Witnessed & Understood by me, Serge Kobele | Date | Invented by | Date

Recorded by David K Clarke.

000089

From Page No.__

Took off PCR mixtures - held on ice until ready to run gel

Prepared a 1% Agarose gel - loaded samples

LHS 1Kb Ladder & 1µg / Space / (+) Nuclease / (-) Nuclease /

loaded 10µl of PCR RXN mixture which contained some 'gunk' at the mineral oil/H_2O interface.

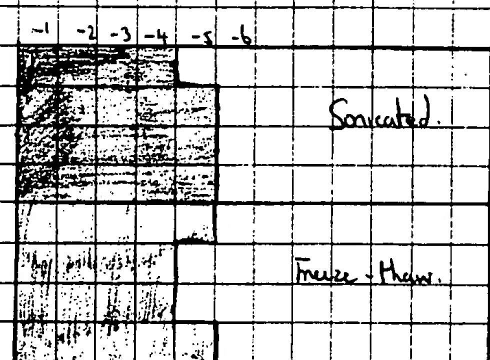
1Kb Ladder

5090
4072
3054
2036
1636
1018
506/517
396

(MAB-13-1 is on it's way from Bruce Fernie and hybridoma cells will be available later). It reacts to an epitope in Antigenic site B. It does not neutralise the Aus/02/61 strain, but will neutralise Mass/9320/77 (Subgroup B virus)

cDNA/PCR RXN did not work. Should probably run a Flu cDNA synthesis reaction control.

Did final read on $TCID_{50}$ for Sonicated vs Freeze-thaw



Not much difference

Will culture Veros into a fresh TC150 x2 - will do an exp on monday to assay virus production in a timed manner

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Witnessed & Understood by me, [signature] | Date | Invented by | Date
Recorded by [signature]

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From Page No. 67

Passed Vero cells into 2 x 160 cm^2 bottles – will infect one bottle on monday with 1ml of working stock virus

Checked 2 subcultured Vero clones – they are doing fine – will check again on monday.

B-subgroup Virus will soon be on it's way

Will ask Kim to break out Hep-2 cells and adjust one of the incubators to 32.5°C.

To Page No.____

Witnessed & Understood by me, Sig W. Kl[illegible]

Date

Invented by

Date

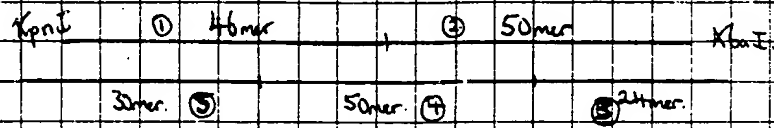
Recorded by David K Clarke

From Page No.

Prepared stock x10 Acetate electrophoresis buffer and 50% glycerol BPB in H_2O

Redesigned Oligos for TaiAn - into ≈ 50mers

for the Leader Region



46mer ① 5' CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA AAC TTG CAT
✓ AAAC ordered

50mer ② 5' CAA AAA AAT GGG GCA AAT AAG AAT TTG ATA AGT ACC ACT TAA ATT
✓ TAA CT ordered

24mer ③ ✓5' CTA GAG TTA AAT TTA AGT GGT ACT ~~TAT CAA AT~~ ordered

50mer ④ 5' TAT CAA ATT CTT ATT TGC CCC ATT TTT TTG GTT TAT GCCA
✓ AGT TTG TTG TA ordered

30mer ⑤ ✓ 5' CGC ATT TTT TCG CGT AAT ATG CGT CGG TAC
ordered

Oligos also have to be designed for the trailer region.

P.T.O

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Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Khle		Recorded by David K Clarke	

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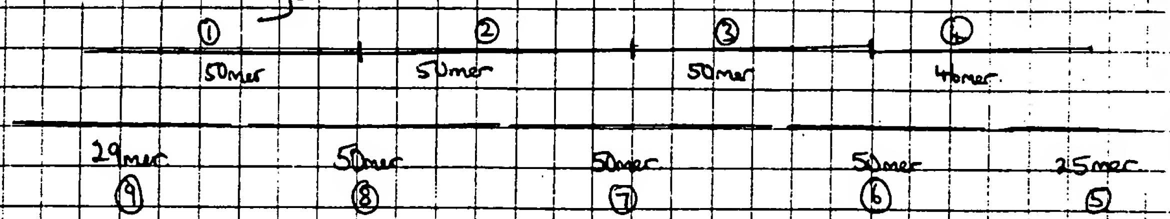
Trailer Oligos.



✓ ① 5' GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA TAA TTTTTT
50mer AAA TA

ordered

✓ ② 5' ACT TTT AGT GAA CTA ATC CTA AAG TTA TCA TTT TAA TCT TGG AGG
50mer AAT AA ordered

✓ ③ 5' ATT TAA ACC CTA ATC TAA TTG GTT TAT ATG TGT ATT AAC TAA
50mer. ATT ACG AG

ordered

✓ ④ 5' ATA TTA GTT TTT GAC ACT TTT TTT CTC GTT ATA GTG AGT CGT
46mer. ATT A

ordered

✓ ⑤ 5' AGC TTA ATA CGA CTC ACT ATA ACG A
25mer. ordered

To Page No.

Witnessed & Understood by me, Date Invented by Date

Segi W. Kohl

Recorded by David K Clarke.

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From Page No. 70

✓ ⑥ 5' GAA AAA AAG TGT CAA AAA CTA ATA TCT CGT AAT TTA GTT
50mer AAT ACA CAT AT
ordered

✓ ⑦ 5' ~~AAA CCA ATT AGA TTA GGG TTT AAA TTT ATT CCT CCA AGA TTA~~
50mer AAA TGA TAG
ordered

RSV-CAT Leader-7
Should actually be Trailer

✓ ⑧ 5' ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT GAA AAA TTA TAT
50mer GAT TTT TA
ordered

✓ ⑨ 5' ATT TTT AAT AAC TAT AAT TGA ATA CTG CA
29mer
ordered

If RSV infectivity is largely associated with cellular debris, then can use that fact to concentrate the virus - however may need to plaque pick and/or go to Hep-2 cells

To Page No.______

Witnessed & Understood by me, Date Invented by Date

Serge W. Klein

Recorded by David K. Clarke

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From Page No.

Checked Vero Cells - will pass and infect today - Infect at 10am with P_3 Vero (working stock - 1ml) - will remove inoculum after 3hrs adsorption.

Vero clones are doing fine - will sub-culture 3 more today - and feed the others tomorrow.

10am Initiated infection using 1ml of working stock p_3 Vero on the TC150. adsorbed 3hrs - then added 20mL of MEM and replaced at 37°C

Asked Kim for Hep-2 cells. Adjusted one incubator to 32.5°C for temp. exp.

Set up 2 x 25cm flasks for Wed to carry out time course experiment - will harvest at 24, 30, and 48h p.i. (2 x 1.5ml) - will leave 1ml aliquot untreated - will spin the other in the microfuge and assay the supernatant for infectivity. (will try to resuspend pellets in 150μl MEM and assay that)

Will need 4 x 96 well plates for Fri

Sub-cultured 3 more Vero clones into 24 well plates

Will ask Ying for 'Cat plasmid' and book PCR machine as soon Tai Ah delivers the oligos

Checked all oligos needed for production of RSV ends for CAT work will order this afternoon.

Ordered 100g of Na Deoxycholate from Sigma - should arrive by weeks end

Prepared 1litre of 10x Versene solution and 1Litre of 10x Hepes/NaCl/$MgCl_2$ (as hypotonic resuspension buffer

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Witnessed & Understood by me, Date Invented by Date

[signature]

Recorded by David K. Clarke.

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From Page No.

Checked infected cells — will start harvest at 10am — there ~~are~~ is already a pronounced c.p.e. but not many cells floating in the medium.

* NB Density of Ncapsid 1.31 — 1.32 $g\,cm^{3^{-1}}$

Estimate 100s for Ncapsid S-Value (not necessary)

Was able to calculate ~~time~~ run length for SW 28 at 25K to give equivalent sedimentation value as SW41 spin.

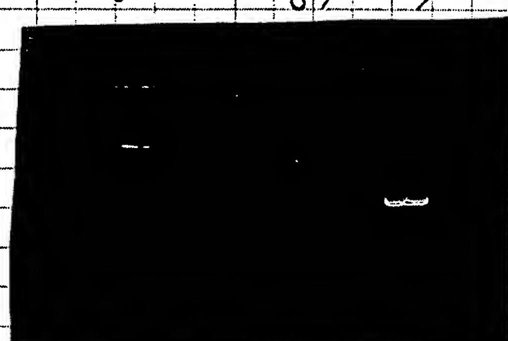
Spin length/conditions are 3hrs 50mins at 25K, 12°C in SW28

For cell harvest (see above) Collected 3ml into 50µl Hepes/50µl $MgSO_4$ — mixed and aliquoted 1.2ml into each of 2 eppy tubes. Spin one tube at 4°C 10K×g for 5mins, (did nothing to the other) Harvested super from spin tube and froze both samples in an EtOH/dry ice bath.

12pm put on restriction digest Hind III on CAT plasmid given to me by Ying.

5µl ×10 buffer.
2µl plasmid (≈ 9µg)
43µl H_2O
50µl + 1µl Hind III (20u µl^{-1})

Analysed 2µl on a 1% Agarose gel with ladder to check it was cut
(Ruth ran the gel).



Hind III cut CAT ≈ 4Kb Plasmid (Ph)

Uncut plasmid may pose a background problem, will El. El. PCR product

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[signature]

Recorded by David K Clarke

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From Page No. 73

~~Wed~~ Completed 30hr harvest from RSV p3 Vero infected cells — spun one eppendorf and left the other as is. Froze both and stored at -80°C.

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Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kble		Recorded by David K Clarke	

From Page No.___

Sent Fax to John with Paper Corrections etc.

10am - Carried out harvest of virus at 48h p.i. - Spun one sample as before and retained supernatant - froze down both aliquots. Also scraped monolayer (which was showing complete fusion but not completely disintegrating) into remaining MEM (≈ 10ml) - Pelleted - 4°c 3K rpm 10 mins - poured off super and resuspended pellet in 1ml MEM/Hepes/$MgSO_4$ - Freeze-thawed - respun at 1.5K for 5mins and collected the super - froze at -80°c

Checked Vero clones - 2 more clones will be ready for sub-culture tomorrow. Will feed 2 of the clones in the 24 well plates (and pass them on Thur or Fri)

prepared another series of RXN buffers for N capsid preparation.

X10 Tris/NaCl/EDTA pH 7.5

10% SLS

40% Sucrose

programmed PCR machine for Rescue of CAT gene from Flu construct.

1min at 94°C

2mins to cool to annealing temp

2mins at 49°C Annealing temp

1min to heat to RXN temp 72°C

3mins at 72°C

1 min to get back up to 94°c

Infected 2 x 25cm² flasks - each with 300µl of RSV p_3 Vero - adsorbed one at 37°c for 2-3hrs and the other at 32°c for 2-3hrs

Set up PCR RXN - collected Hind III cut CAT plasmid

70% EtOH washed - Dried

Resuspended in 7µl H_2O

Used 1µl for PCR RXN

Ordered forceps from VWR.

See Overpage

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Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K Clarke

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From Page No. 75

7µl (1µg) DNA
1.5µl Each primer (100ng µl⁻¹)
2µl dNTPs (10mM)
5µl x10 Rxn buffer
38µl H_2O.
49µl — Heated at boiling for 2mins snap cooled spun briefly added 1µl of Taq pol. overlaid with mineral oil and started PCR Rxn.

To Page No.

Witnessed & Understood by me, Sage W. Klebe

Date

Invented by

Date

Recorded by David K Clarke.

Project No._______

TITLE ______________________________ Book No._______

From Page No.__

Took off PCR Rxn, prepared 1% Agarose gel and analysed 5µl of PCR products alongside DNA 1Kb ladder.

If Rxn has worked will Phenol Chloroform extract, EtOH ppt, digest with Xba I / and Bst I gel purify the fragment

Oligos need to be gel purified and Kinased (except for those with free 5' ends).

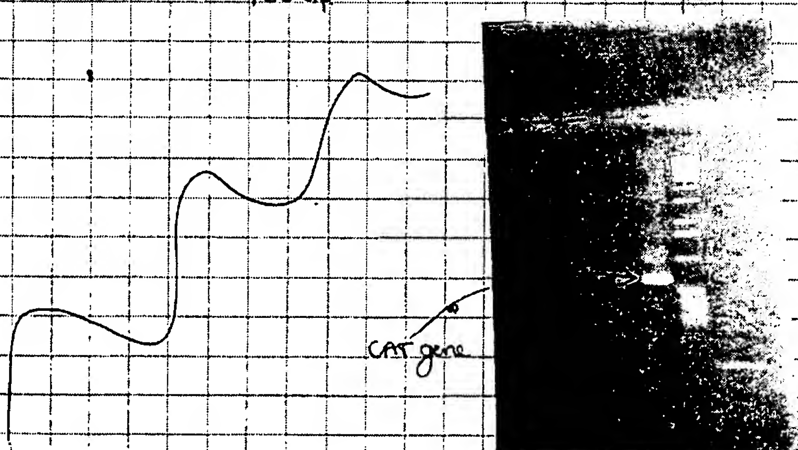
Checked infected cells 32°C/37°C – No significant differences in c.p.e – although both monolayers do look 'infected.'

Ordered Pheno/chloroform/Isoamyl pH 6.7 and Chloroform/Isoamyl
25 : 24 : 1 24 : 1

Prepared a 60% w/v soln. of CsCl in H_2O – Density = 1.46 g.ml^{-1}

When making CsCl gradient will put ½ of the 40% layer as 60% to ensure N. capsid doesn't pellet through.

Photographed amplified CAT gene – looks pretty good ≈ 1-2µg total CAT gene DNA (size ≈ 700 b.p.



Completed Ø chloroform extraction and EtOH ppt^{ed} o/n at -10°C.

To Page No.____

Witnessed & Understood by me, Date

Invented by Date

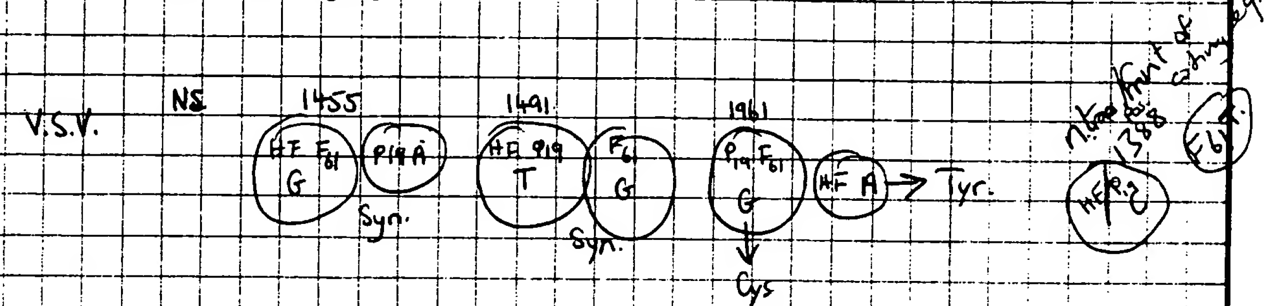
Recorded by

000100

Project No.______
Book No.______ TITLE ______

From Page No.___

Checked infections at 32°c and 37°c. - c.p.e is v. advanced at 37°c but not so advanced at 32°c



Subcultured [redacted] Vero clones 1 and 2 into 6well plates in duplicate, also subcultured one more clone from initial 96well plate into a 24well plate (however only a few cells made it through the transfer - probably because the cells had been growing for a long time and were not firmly fixed to the plastic)

Will carry out XbaI digestion at 37°c for 1-2hrs in 20µl - followed by PstI digestion at 37°c in 50µl.

Set up XbaI digest.

2µl x10 buffer
1µl [redacted] BSA (1000µg ml^{-1})
16µl H_2O
1µl Enz
20µl 37°c 1.5hrs

Then added 5µl x10 PstI rxn buffer + 24µl H_2O and incubated at 37°C for a further 2hrs.

Meanwhile, set up 4 $TCID_{50}$s to compare virus titers for P_3 Vero 24hr, 30hr, 48hr harvests - spin/not spin and for freeze-thawed cellular material x10 conc after 48hrs

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Witnessed & Understood by me,	Date	Invented by	Date
Suzy W. Kble		Recorded by David K Clarke	

000101

Project No.

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From Page No. 78

Harvested virus from 32°C / 37°C infections. Cell monolayer at 37°C was blown away (72h p.i.). The cell monolayer at 32°C was completely syncytiated but was not falling apart as at 37°C.

Resuspended cell debris pellets in 1ml of MEM.

Froze down 3 x 1ml aliquots from each harvest.

Checked conc. of NP-40 supplied (in stockroom)

Made up 10% Na Deoxycholate (100ml)

Also prepared 5M NaCl (500ml)

1M Tris (1L) pH 8.0.

Need to call Sigma for N-P 40 conc.

To Page No.

Witnessed & Understood by me, Syl W. Kbl

Date

Invented by

Date

Recorded by David K. Clarke

Checked Vero cells to be infected with RSV for Nucleocapsid.
Will leave them until tomorrow.

Checked $TCID_{50}$ - although results are preliminary it looks as though similar amounts (but v low) of virus are released into the supernatant from infected veros at 24 and 30 h.p.i. These virus appear to be free-floating and not associated with cellular debris at these "early stages". At 48h.p.i a greater percentage of total infectivity appears to be cell associated. Also at 48h.p.i the majority of infectivity can be pelleted at 3,000 rpm and released from the cell pellet by one cycle of freeze-thaw in 1ml MEM (≈ 100 fold more virus appears to be cell associated at 48h.p.i vs free floating.

Will shoot for N.capsid prep^n at ≈ 30h.p.i

George prepared a low M.P Agarose gel - will run my trimmed CAT gene

1000g ≈ 2200 r.p.m. on Table top centrifuge.

Irradiated gel with Long wave U.V light (to minimise U.V. damage) - there appeared to be 1-2µg of DNA in the appropriate band - excised this from the gel - melted down at 65°C for 10-15 mins added 1/10th vol (35µl) of 10X Agarase buffer and 2µl of Agarase and placed at 42°C for 1hr. Then added 1/10 vol 3M Na Acetate, mixed and held on ice for 15mins, then spun for 15mins and took supernatant; added 2vols of Isopropanol - held on ice for 5-10 mins, then spun down for 10mins - EtOH (70%) rinsed pellet and dried in speed vac.

resuspended dried pellet in 50µl H_2O and analysed 5µl on a 1% agarose gel.

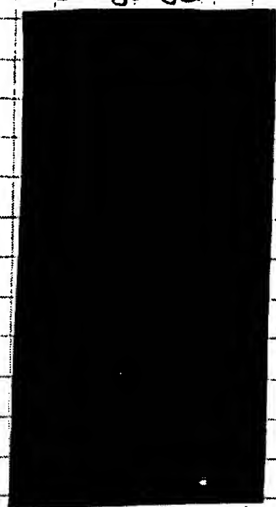
Checked Vero clones - Will sub clone No. 3 into 6 well plates tomorrow, and will infect 1 and 2 with RSV (may need to subculture into 25cm² Flask).

Witnessed & Understood by me, Serge W. Klele | Date | Invented by | Date

Recorded by David K Clarke

From Page No. 80

George's Gel



Gel analysis of Xba/PstI trimmed and gel purified CAT gene

Reran 1.5µl of purified DNA to check if the 'trimmed' DNA species is actually one DNA species as there is some doubt from this gel.

Ran 1µl of Marker 1Kb ladder as well.

2nd gel shows that there is only one species of DNA of the correct size (the Cat gene).

Based on this (and the above gel) it would appear there is ≈ 2 - 2.5µg of this DNA.

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Witnessed & Understood by me, | Date | Invented by | Date

Recorded by

000104

Project No.______
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From Page No.__

See reference binder #22A

9am Infected 10 TC 150s, each with 1ml of P_3 Vero (working stock)

Adsorbed at 37°C for ≈ 3hrs (Kept 10 bottles as control)

Also infected 1x25cm² flask of Hep-2 cells with 300µl of working stock.

Fed each bottle with ≈ 10ml of MEM.

Checked $TCID_{50}$ – looks as though very few virions are budded out from the cells – even at 48h p.i. – Most virions appear to remain associated with the cellular debris and cells even when the cell monolayer is essentially wiped out.

RSV subgroup B virus arrived from Mike Hendry (strain 9320) – stored at -80°C in Box with Judy Beeler's stuff.

Subcultured Vero clone N° 3 into a 6 well plate. Subcultured clone 1 into a 25cm² flask. Also infected Vero clone ① in the 6 well plate – added 100µl of [redacted] P_3 Vero 'Working stock' and adsorbed for ≈ 2.25hrs at 37°C.

Then added 1-2ml MEM and continued incubatⁿ for 48hrs

Ordered 250ml centrifuge bottles from Beckman.

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Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K Clarke

TITLE: Oligo Purification à la Tai An. Project No._____ Book No._____

From Page No.__

Checked infected TC150s - They are showing the beginning stages of c.p.e if c.p.e are not sufficiently advanced by 3pm - will place at 32°c O/N.
To start harvest.

Checked infected Vero Clone ① - No obvious c.p.e yet - will check again at 6pm.

Checked Vero subclones ② - they are ready for

Purified 5 leader Oligos according to the protocol of Tai An.

① 5ml Methanol
② ≈10ml H_2O.
③ load Oligo (in elution buffer - gel slice) ≈ 5ml
④ Reload Oligo ≈ 5ml
⑤ Rinse with Oligo buffer 5ml
⑥ Rinse with H_2O ~ 5ml
⑦ Elute in 3ml of 50% Methanol.

1 drop sec⁻¹

Dried down in speed vac.

Placed infected TC 150s at 32°c to slow down development of c.p.e. Will place at 37°c tomorrow morning prior to N capsid harvest.

Checked Hep-2 infected cells - a little c.pe is apparent - will check again this evening and again tomorrow morning.

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Witnessed & Understood by me, Sage W. Kh[illegible] | Date | Invented by | Date

Recorded by David K Clarke

Project No._______

Book No._______ TITLE _______

From Page No. 83

8 Prepared x1 Versene, x1 Hepes/NaCl/$MgCl_2$,

40% CsCl ≡ 24ml 60% CsCl
3.6ml x10 buffer (Tris etc)
750µl S.L.S (10%).
to 36ml with H_2O

30% CsCl ≡ 18ml 60% CsCl.
3.6ml x10 buffer.
750µl S.L.S
to 36ml with H_2O

25% CsCl ≡ 15ml 60% CsCl
3.6ml x10
750µl SLS
to 36mls with H_2O.

5% Sucrose - 5mL (40% Sucrose)
4ml x10 buffer (as above)
80µl SLS
to 40ml with H_2O.

Infected Vero clone (2) with 0.1ml of RSV P3 Vero (working stock). Adsorbed ≈ 2.5hrs.

Also passaged duplicate Vero clone (2) into a TC 25 cm^2.

Checked growth of Vero clone (1) in 25cm^2 Flask.

Measured O.D.s of purified Oligos which were resuspended in 100µl sterile H_2O
(Used 5µl + 995µl H_2O) - See Oligo folder for details.
of stock
All were at least 70µg total in the 100µl aliquot.

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Witnessed & Understood by me, Serge W. Kirk | Date | Invented by | Date

Recorded by David K Clarke

From Page No. 84

~~Checked~~ – Vero Clone 1 in $25cm^2$ Flask is growing well – will probably freeze down on Friday. Clone 3 is growing well in 6well plate – will split into Two 6well plates on Fri. and feed 4, 5 and 6 as they are very sparse – will use fresh G. medium from Kim.

To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
Inge W. Kelle		Recorded by David K Clarke	

Project No._______

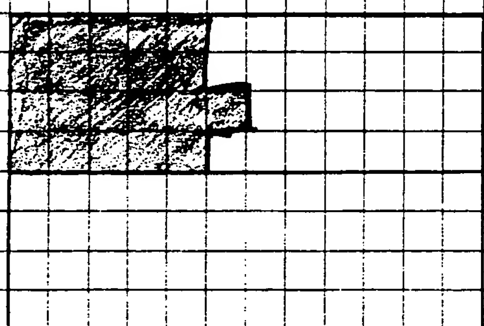
Book No._______ TITLE ____________________

From Page No.__

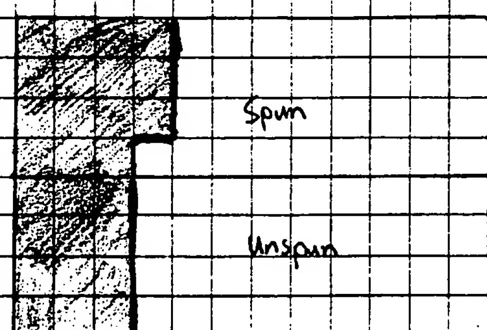
Checked infected Vero Clone (1) - c.p.e. Now apparent - will harvest this afternoon or tomorrow - Hep-2 cells are also looking pretty sick - will leave until this afternoon or tomorrow.

Checked infected TC 150s - on 5 of flasks cpe looks very nice - lots of syncytia, on the other 5 cpe is not as extensive - placed all 10 at 37°C until 10am ∴ (1.25hrs)

Carried out N.capsid extraction procedure.



Freeze Thaw Samp x10 conc. 48h.p.i.



24h.p.i.

May be some contribution from added inoculum - needs to be retested after removal of inoculum

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Witnessed & Understood by me, Serge W. Kibble

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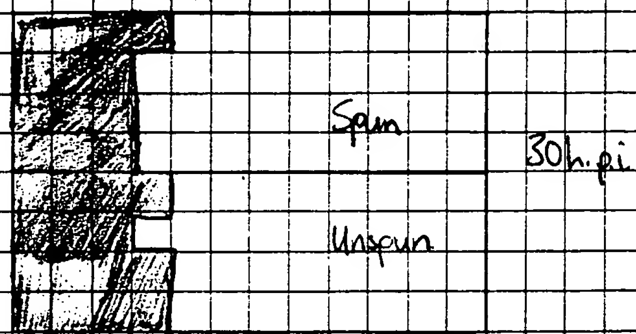
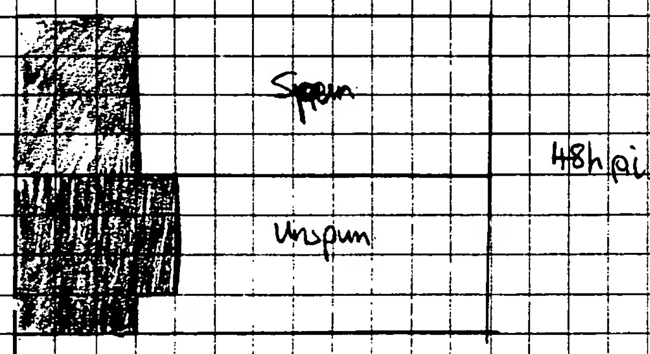
Invented by

Date

Recorded by David K Clarke

From Page No. 86





More and more virus is associated with cellular debris at this point in the infection.

Harvested Virus from Vero Clone ① infection – managed to remove most of the cell monolayer by pipetting – added 100µl Hepes/30µl $MgCl_2$ – went through one freeze-thaw cycle without spinning – aliquotted and froze.

Set up o/n bacterial cultures (Top 10 F' – invitrogen) containing pUC 19 – Streaked colonies on Amp^+ Agar (used 100µg ml^{-1} in liquid broth) –

Checked CsCl gradients after spin – Nothing was visible on either Control or infected cell lysates.

Harvested Virus from Hep-2 cells – saved 1ml for 5min microfuge spin etc.

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From Page No.__

Checked c.p.e on Vero Clone 2 infected with P_3 Vero (100µl) – V. extensive – May indicate this Vero clone is v. susceptible to RSV.

Carried out Alkaline Lysis technique for mini preps – used ≈ 6ml total volume of φμ bacterial culture.

Stored final N.acid ppt. as dried pellets at –10°C. – Ran 3-4µl of 20µl from ¼ (1 tube) of total prep. Loaded ≈ 2/3 of that sample.

There appeared to be ≈ 200–500ng of DNA on the gel – so can use ½ of total prep – will purify on a low mp gel first – then cut with suitable/appropriate enzymes

Took advantage of 5xTC150s That Kim had as extras and infected each with ≈ 0.7ml of ATCC P_2 Vero (m.o.i of ≈ 0.001) – will leave over the weekend, and harvest on Mon/Tues.

Meanwhile froze down Vero clones 1 and 2 – Resuspended cell pellets in 90% FCS 10% DMSO after trypsinization and dilution in Growth medium.

Fed Vero clones 4, 5 and 6 with some fresh growth medium

Passaged Vero clone 3 into 2 x 6 well plates

Set up $TCID_{50}$s on 32°C/37°C P_3 Vero infected
Hep2/Hep2 microfuged 5mins P_3 Vero inf.
Vero clones 1/2 P_3 Vero infected

Stored Vero clones in Upright freezer.

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Witnessed & Understood by me, | Date | Invented by | Date

Serge W. Kbl

Recorded by David K Clarke

Project No.

TITLE: Book No.

From Page No.

Informal interview with (Rong-guo Qiu) Ron Chiu

Very pleasant fellow, and well experienced in mol. biology.

Checked infected Vero's - They are already showing fairly good c.p.e. - It seems that slightly elevated temperature is better of RSV growth etc.

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Witnessed & Understood by me, Date Invented by Date

Sergio Kobbe

Recorded by David K Clarke

Project No.

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From Page No.

Preliminary conclusions from TCID$_{50}$s

① Hep-2 cells bud virus out better than Veros.

② The new Vero clone ① looks to be producing considerably more virus than the standard Vero cells.

Reconditioned SW41-GAF from Beckman

Checked infected TC150s — They were blown away – there were no areas of cells remaining unaffected by virus cpe.
Harvested as per usual – total # 90 mL as (P$_3$ Vero ATCC 11-22-93) froze down.

Checked Vero clones — Clone ③ is ready to be infected and passaged.

Infected V.Clone ③ with 100 µl of P$_3$ Vero (most recent from 5 x TC150s).

Also passaged V. Clone ③ into a 25cm^2 Flask.

adsorbed ≈ 3hrs at 37°C then added 2ml MEM.

Set up Kinasing reaction on oligos 187, 188, 189.
Used ≈ 5µg of each oligo

1mM Spermidine
200 µM ATP
X10 RXN buffer
1µl Enzyme in final vol. of 50 µl.

1hr at 37°C (actually 1½ hrs!)

42
32
74

Am leaving Leader ① and Leader ③ unkinased for ligation purposes.

After Kinase RXN, phenol chloroform extracted and EtOH ppt^{ed}.

~~Prepared x1 TE buffer – autoclaved~~

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Witnessed & Understood by me, Date Invented by Date

Serge W. Kable

Recorded by David K Clarke

From Page No. 90

T_m for annealing of 1 and 5 (leader oligos) is 76°C ✓

T_m for annealing 4 to 1-5 is 52°C

T_m for annealing 2 to 1-5-4 is 76°C

T_m for annealing 3 to 1-5-4-2 is 50°C

Therefore will anneal 2 to 4 separately and 1 to 5 separately to accommodate higher T_m's. Will then mix both together and anneal, finally adding 3 to complete the RSV end — Then ppt and ligate

equimolar amount (use less) ≈ ½ amount

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Witnessed & Understood by me, Serge W. Klh | Date | Invented by | Date

Recorded by David K Clarke

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From Page No.

Prepared 1% L.M.P. Agarose gel ~~preparation~~ to cut out pUC 19 and gel purify.

Checked P3 Vero infected Clone 3 — no sign of c.p.e. yet.

$TCID_{50s}$ are still showing 1 a better yield with Vero clones 1 and 2 than with the usual Vero cells.

2 Much better yields from Hep-2 cells and budded out from the cells much better.

Spun down some of Kathy's Vaccinia DNA — will cut with Bam HI

Lost 'F' protein in speed Vac — resuspended 'M' in 70% etOH added NaCl and re ppt at −10°C in order to get it into eppy tubes

Spun down 5mins — in microfuge — dried — resuspended in 50μl H_2O

Set up Digest 25μl of 'M' DNA solution.

5μl x10 buffer

5μl BSA 1mg ml^{-1}

12μl H_2O

3μl Enzyme

50μl — 2hrs at 37°C

Ran Puc 19 on a L.M.P. agarose gel — Excised Band of DNA and ran through β-agarase extraction procedure

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From Page No.__

Vaccinia M clone cut with Bam H.I

Smear of vaccinia DNA + host DNA and some RNA.

tRNA ?

Will probably have to do a PCR RXN to amplify up the appropriate genes

However - will try to purify genome first

Completed purification of ~~..~~ PuC19 DNA. - stored at -10°C as dried pellet - will resuspend at 100ng μl^{-1} i.e. ≈ 50μl.

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Witnessed & Understood by me, Gary W. Klein | Date | Invented by | Date

Recorded by David K Clarke

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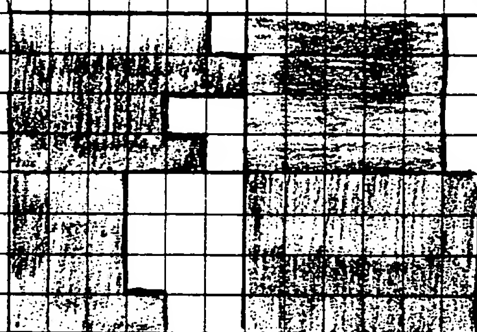
From Page No.__

Did final Readings on $TCID_{50}$. Results are very interesting.
All infections were with "Working stock" - ATCC P_3 Vero virus.

32°C
$10^{5.5}$

37°C
$10^{3.75}$



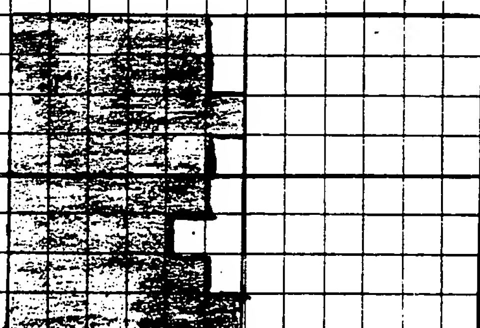
Hep-2 spun 10,000xg 5min
$= 10^{5.5}$

Hep-2 total
$= 10^{6.5}$

Will Reset incubator at 32.5 and do Hep-2 experiment at 32°/37°

Vero Clone 1
$10^{5.75}$

$10^{5.5}$
Clone 2



Ordered autoclaveable cell scrapers (Bellco)
* Disposables (Fisher)

Froze down Vero clone 3 with clones 1 and 2

Fed clones 4 - 5 and 6.

Harvested virus from Clone 3 infected with RSV ATCC P_3 Vero
After scraping cells (didn't get them all!) into suspension - did a freeze-thaw and then spin down and aliquotted super prior to freezing.

C.P.E could have been a little better (but they were pretty good)

To Page No.____

Witnessed & Understood by me, [signature] | Date | Invented by | Date
Recorded by [signature]

From Page No.__

Set up digestion of PUC 19 (gel purified) - started out with Kpn I digestion in 30μl final vol.

1.5μl ENZ.
3μl x10 buff.
3μl x 10 BSA.
22.5μl H_2O
30μl

2hrs at 37°c (12-45pm start)

Oligo annealing -

Will do annealings in 0.1M NaCl, 10mM Tris, 1mM EDTA at appropriate T_{Ms}. (Use small volumes - 30-60mins per annealing) 20-50μl at final stage

Will resuspend each oligo (Kinased) in 10μl of H_2O.

Checked Vero clones - threw out 6 (appeared to be some contamination and the cells were not growing very well) Clone 5 appeared to be growing fairly well - so can probably split into 2 x 6 well dishes by tomorrow or Wed.

Heat inactivated KpnI at 68° for 10-15mins - then set up Hind III digestion in 50μl using 1.5μl enzyme.

Prepared a LMP 1% agarose gel and ran double digested PUC 19 DNA alongside a 1Kb DNA ladder - Excised ≈ 3Kb band and did agarase treatment.

Noted that there appeared to be a blurred or possibly doublet in the band of digested DNA - will run another gel tomorrow (regular 1%) and test a small sample of the eluted DNA. (About 1/10th should be enough).

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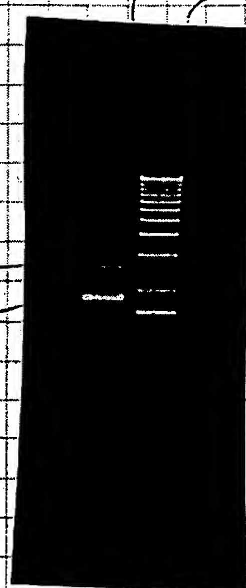
Prepared a 1% agarose gel for analysis of gel purified KpnI/HindIII cut PUC 19. Run $\frac{1}{10}^{th}$ of the purified material.

Infected v.confluent Hep.2 cells with 0.25ml of 'old' P_3 Vero (ATCC) for 32°C/37°C experiment - adsorbed both for 2-3hrs at 37°C then placed the "32°C flask" at 32°C

Meanwhile carried out $TCID_{50s}$ on Vero clone (3) Virus (V.clone (3) infected with P_3 Vero old)

also did $TCID_{50}$ on 'New' Working Stock, P_3 Vero.

Split Vero clone (5) into 2 separate 6 well plates



photographed digested puc 19 - it's not cutting efficiently!

will do digestion on mini prep DNA and EtOH ppt in between digestions

Spun down Kinased Oligos - did not see a visible "pellet" in (189) will add more salt + glycogen to ensure ppt. Meanwhile dried the other two oligos and stored at -10°C.

Will add some more NaCl and try spinning down again tomorrow

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Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

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TITLE: Book No.

From Page No. 96

Will set up Kpn I digestion on remaining mini prep DNA in 80μl final vol

8μl x10 buffer
~~8.9μl~~
8μl of 1/10th diluted BSA stock
61μl H_2O
3μl KpnI
80μl (½μl RNase A - optional)

Ran control digestion in 20μl.

Phenol Chloroform extract - ppt at -10°c o/n and do Hind III digest^n tomorrow.
Retained 4μl for gel analysis.

~~Will~~ Will anneal $\boxed{11 \text{ and } 5}$ as follows - Mix 5μl of 190 (unkinased) with 2.5μl
of 187. 76°C for 10mins - let cool slowly to 60°C - hold for 10mins - let cool
to 50°C → ice.

Respun 189 - looks as though some DNA came down - put back at -10°c o/n.
Will spin again tomorrow.

NB Should try an infection of New Vero clones with a higher titer virus stock e.g.

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Witnessed & Understood by me, Date Invented by Date

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From Page No.__

Spun down Kpn I digested pUC 19 miniprep DNA – set up Hind III digestion in 80µl

8µl x10 buffer
69µl H_2O
3µl Enzyme
80µl

Set up Hind III control with Georges plasmid

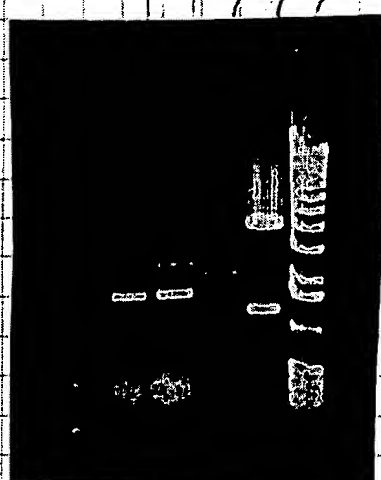
Checked Infected Hep-2 cells; they are already showing some signs of c.p.e. Will leave at least another 24hrs. The level of c.p.e at 32°C and 37°C appear to be similar, perhaps slightly greater at 32°C.

Need to Re-Kinase (189) – Do 10µg this time and add glycogen

Run a 0.7% Agarose gel to check the digestion products

LHS. 1Kb Lad / Kpn Con / Hind III con / Kpn-puc19 / Kpn-Hind III-pUC19/

Hind III cut, Kpn I cut, Hind III con, Kpn I control, Ladder



KpnI/nor Hind III appeared to cut

Ordered 25µg of pUC19 from Pharmacia to solve the problem.

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Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

Project No.

TITLE: Book No.

From Page No. 98

Set up an NcoI digestion on Mini prep DNA which wouldn't cut with KpnI and HindIII, to see if there is a 2nd contaminating plasmid in the mini-prep.
i.e. there is no NcoI site in Puc 19 and there is in the most likely contaminating plasmid.

Final check on infected Hep's – cpe is progressing v well at both 32°C and 37°C.

To Page No.

Witnessed & Understood by me: Syl W. Kbl

Date

Invented by

Date

Recorded by David K Clarke

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From Page No.__

Checked infected Hep-2 cells ; cpe has become extensive, will harvest later this afternoon or tomorrow morning.

Meanwhile set up Kinasing reaction on 189 for the 2^{nd} time.

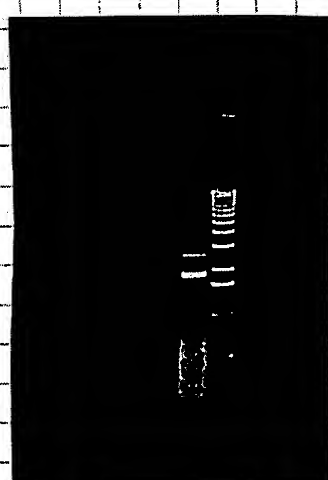
5μl x10 buffer
3μl of 15mM spermidine (1mM final)
1μl of 10mM ATP (200μM final)
12μl Oligo (10μg total)
28μl H_2O
1μl Enz

50μl 1hr at 37°C

Phenol Chloroform extracted and stored at -10°C until Glycogen arrives.

Prepared and ran a 1% Agarose gel to analyse NcoI digestion products (from Mini prep DNA.

Checked Vero clone - will leave until Mon - then will refeed.



Gel shows same DNA pattern for NcoI, as for Hind III and KpnI.

Could be the DNA is modified or a potent inhibitor is present - can design an experiment to test this - add 60μl of Mini prep DNA to 8μl x10 buffer 10μl of DNA ($1\mu g\,\mu l^{-1}$) + 3μl enz.

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Witnessed & Understood by me, Date
Invented by Date
Recorded by

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From Page No. 100.

Harvested infected Hep-2 cells + Added Hepes/$MgSO_4$ after scraping cells into suspension; then went directly to freeze-thaw, followed by a spin — stored the super in 1ml aliquots at -80°C after freezing in EtOH/Dry ice.

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Witnessed & Understood by me, Date | Invented by | Date

Serge W. Keller (signature)

Recorded by David K Clarke (signature)

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From Page No.__

Checked $TCID_{50s}$ - it appears that the 'New' working stock is not V. good (perhaps the infection went too long and a lot of virus lost viability)

Also the titer from Vero clone (3) appears to be fairly good ≈ 10^5 but not as good as clones (1) and (2)

Set up $TCID_{50}$ for Hep-2 32°/37°C experiment - Plated out to 10^{-7}

Carried out purification of the Five oligos which Tai An cut out of a gel - Dried down ....

ppted 5μg of PUC-19 (from Pharmacia) for digestion with KpN I (Put at -80°C for 1hr)

(~~Spun down~~ re-Kinased 189)

Ordered Dounce Homogenizer set; D Stec ordered gradient makers.

Need to copy Ward et al (N.sapid/genomic RNA prep) at UCSF.

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Witnessed & Understood by me, Sergei W. Kibb[?] | Date | Invented by | Date

Recorded by David K Clarke

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Spun down 5μg of pUC 19 (Pharmacia) and set up Kpn I digestion - 2hrs at 37°c in 50μl final vol.

5μl x10 buffer
5μl x10 BSA
38μl H_2O
2μl enzyme

50μl.

Then Phenol chloroform extracted and EtOH ppt.ed

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[signature]		Recorded by David K Clarke	

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Did final $TCID_{50}$ reading on Vero Clone 3 and New P_3 Vero ATCC working stock

-1 -2 -3 -4 -5 -6

$10^{5.25}$ Vero Clone 3 infected with P_3 Vero ATCC (Not as good as clones 1 and 2)

$10^{5.5}$ 'New' 'P_3 Vero working stock' ATCC - comparable to 'Old' P_3 Vero working stock - will use this for a N capsid prep/virus prep next MON or the following MON.

Set up Hind III digestion on Kpn I digested PUC19 (Kept 1µl of Kpn I cut material for gel analysis)

5µl x10 buffer
43µl H_2O
2µl enz
50µl 2hrs at 37°C. off at 12.30pm

Prepared a 1% agarose gel for analysis of digestion products

Infected 1x25cm flask of Vero and 1x25cm² flask of Hep-2 cell, each with 0.5ml of 9320 B virus adsorbed 3hrs (2-15pm feed).

Resuspended Tailer-oligos in H_2O and measured 5µl in 1ml to get an estimate of quantity. (Watch 206 carefully!)

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Witnessed & Understood by me, Serge W. Kobl[illegible]
Date
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Date
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From Page No. 104

KpnI/Hind III pUC19
Kpn cut pUC19
1Kb ladder

Phenol chloroform extracted and EtOH ppt^ed.

EtOH ppted Leader Oligo (Kinased) 189 without glycogen to see if it will come down.

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Prepared low m.p agarose gel for purification of KpnI/HindIII cut pUC19 Vector.
Loaded gel and Ran at 50mA

Set up Kinasing of trailer oligos (except 201 and 197) - left unkinased at ends of trailer.
50µl final vol
~~10µl~~ 10µg of oligo in 6-35µl H_2O
5µl x10
3µl 15mM spermidine
1µl 10mM ATP
H_2O to 50µl
1µl Enzyme.

11.15am - 12.15pm → ØChloroform
EtOH ppt^{ed} (no glycogen)

Spun down Kinased leader (ppt^{ed} yesterday)

Checked Vero clone 15 - Needs to be fed - slow growing - may not be very productive for virus.

Checked c.p.e on 'B' virus infected Veros and Hep-2s - as yet there is no obvious c.p.e on either cell monolayer - tomorrow will be a more 'acid' test.

Continued purification of pUC19 on a gel - phenol chloroform extracted prior to isopropanol ppt. step - spun down, and resuspended in a total vol. of 20µl H_2O and froze at -10°C.

Interviewed Ron Chiu.

See reference binder #22A

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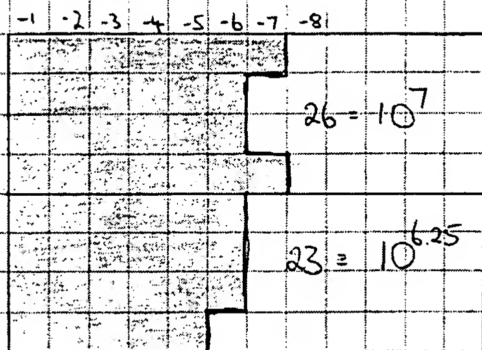
From Page No.

Checked c.p.e on Vero / Hep-2 cells with 'B' virus
There is no obvious c.p.e yet – may change medium tomorrow to see if that will stimulate things

Did final read on $TCID_{50s}$

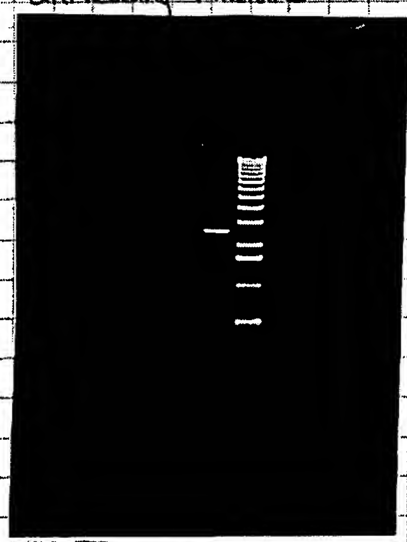


Hep-2 – infected with P_3V 32°C * Highest titre obtained so far with RSV.

Hep-2 infected with P_3V 37°C

Prepared a 1% Agarose gel, and ran out ≈1µl of gel purified PUC19 (from 20µl) to check amount and efficiency of purification etc.

Prepared Leader annealing mixtures



1% Agarose gel of Gel purified PUC 19

50ng ≈ 1/20

≈ 1-2µg of DNA.

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Carried out annealing of leader Oligos — forgot to use Kinased fragments appropriately, but will run on a 1% Agarose gel to check how well they have annealed.

Prepared 1% gel — run — photographed

higher titer stock $\approx 10^7 ml^{-1}$

Meanwhile infected 2 x Hep-2 with 0.25ml of P_3V PH-2 and 0.25ml of "New Working stock" P_3Vero adsorbed 3hrs $\approx$ 6-15pm

Oligo annealing (Leader)

Added 1.5μg of 190 ① and 1.2μg of 187 ⑤ in 10μl (using X10 NTE)

Equimolar ratio.

unkinased

Kinased. (300ng μl^{-1})

Hold at 76°C 10 mins
Cool to 70°C 5 mins
Hold at 70°C 10 mins
Cool to 65°C 5 mins
Hold at 65°C 10 mins
Cool to 60°C 5 mins.
Hold at 60°C 10 mins.
Cool to 55°C 5 mins.
Hold at 55°C 10 mins.
Cool to 50°C 5 mins
Hold at 50°C 10 mins.

Kinased Kinased
Oligos ② and ④ can be
188 189
annealed at the same temperatures

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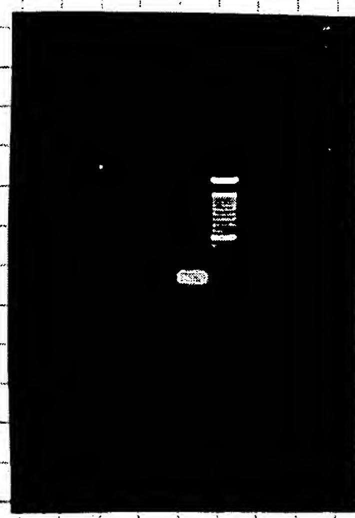
Witnessed & Understood by me, Sigy W. Kibl | Date | Invented by | Date

Recorded by Daniel K Clarke

From Page No. 108

To anneal (1/5) to (2/4) + Oligo (3) 186
unkinased

Hold at 55°C 10 mins
Cool to 50°C 5 mins
Hold at 50°C 10 mins
Cool to 45°C 5 mins
Hold at 45°C 10 mins
Cool to 40°C 5 mins
Hold at 40°C 10 mins



Annealing gel looks pretty good - the Oligo band is at the correct position.

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Checked cells infected with 'B' virus - still no major c.p.e detectable
Changed the medium and incubated at 37°c for a further 24-48hrs.

(NB) Ordered Potassium Tartrate.

Prepared 10ml of boiled glycogen 10mg ml⁻¹

Took 1ml and phenol/chloroform extracted for RNA work.

Prepared a 1% Agarose gel and ran out annealed leader Oligos for electroelution purification. EtBr stained - cut out annealed band and electroeluted into T.E. buffer (salt conc may have been too low) — Phenol chloroform extracted and EtOH ppted with some glycogen. Will check efficiency again tomorrow.

Meanwhile prepared NA45 paper according to protocol; also prepared N.E.T and High salt NET

Checked infected HEP-2 cells - Many cells appear to be dying - will leave another 24hrs before harvesting and doing the $TCID_{50}$s. (More look dead with higher titer input virus).

Autoclaved NET and high salt NET

Trailer oligos

6	= 206	Kinased
3	= 205	Kinased
8	= 204	Kinased
7	= 203	Kinased
2	= 202	Kinased
1	= 201	UnKinased
9	= 198	Kinased
5	= 197	UnKinased
4	= 213	Kinased

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From Page No.

Plan for Trailer annealing.

1 2 3 4

9 8 7 6 5

Resuspend Kinased oligos at 5ug ml^{-1}

* 1:9 annealing T_m = 56°C
1:8 annealing T_m = 52°C
2:8 annealing T_m = 64°C
2:7 annealing T_m = 62°C
3:7 annealing T_m = 62°C
3:6 annealing T_m = 62°C
4:6 annealing T_m = 60°C
* 4:5 annealing T_m = 56°C

* add last in annealing - both together?

Anneal 2, 3 and 7 together in 1st run. Then add 8, and 6 for 2nd run, Then add 4 for 3rd run, then 1 for 4th run and then 5 and 9 for 5th and final run.

Use 1ug of each Oligo

Do 10ug total.

1st run.

Hold at 62°C 10 mins
Cool to 57°C 5 mins
Hold at 57°C 10 mins
Cool to 52°C 5 mins
Hold at 52°C 10 mins
Cool to 47°C 5 mins
Hold at 47°C 10 mins
Cool to 42°C 5 mins
Hold at 42°C 10 mins

2nd Run.

Repeat of 1st Run

3rd Run

Hold at 60°C 10 mins
Cool to 55°C 5 mins.
Hold at 55°C 10 mins
Cool to 50°C 5 mins.
Hold at 50°C 10 mins
Cool to 45°C 5 mins.
Hold at 45°C 10 mins
Cool to 40°C 5 mins.
Hold at 40°C 10 mins.

4th Run.

52°C 10 mins
Cool to 47°C 5 mins
47°C 10 mins
Cool to 42°C 5 mins
42°C 10 mins
Cool to 37°C 5 mins.
37°C 10 mins.
Cool to 32°C 5 mins
32°C 10 mins

5th Run.

Repeat of 4th Run.

Witnessed & Understood by me, Sege W. Klele | Date | Invented by | Date

Recorded by David K Clarke

From Page No.

Checked 'B' infected cells – still no sign of c.p.e. – will leave until tomorrow then harvest and make $TCID_{50}$ measurement

Set up leader annealing once more – this time using Kinased oligos.

Mixed 1.2 (1.5μg) of unKinased 190 with 1.2μg (2.4μl) Kinased 187 added 1μl x10 NTE and 5.4μl H_2O → 10μl. Then ran annealing program #60 as before.

Meanwhile prepared 1% agarose gel for analysis of the annealing products + yesterdays electroelution procedure.

Did likewise for Oligos 2 188 Kinased and 4 189 Kinased.

Then mixed both annealed samples and added 3 186 unKinased and went through program "61" 55°C annealing ---→.

Spun down ½ of electroeluted annealed leader – resuspended in 20μl H_2O, and ran 4μl of that on a 1% agarose gel along with ½μl from annealed mixture (which had been Kinased)

Also harvested Hep-2s infected with V_3H_2 and New V_3 grown At 32°C

$\downarrow$ V_3H_2 $\downarrow$ V_3H_1

Froze down – will do $TCID_{50}$s on Wed

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Witnessed & Understood by me, Serge W. Kible Date

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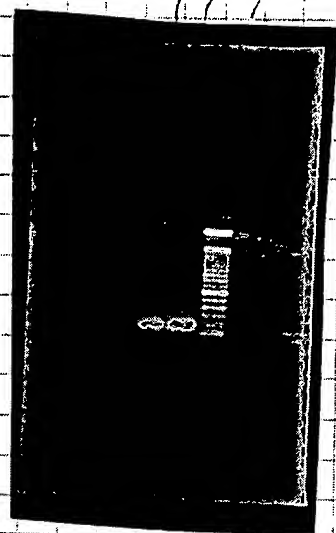
Newer
Older
ladder
100bp ladder



Older annealed oligo. has held up well

looks encouraging.

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Checked c.p.e on B9320 infected cells - may be some on Vero - harvested both Heps and Veros (5 days post infection) - carried out $TCID_{50}$ (out to 10^{-3} dil^n) for both harvests.

Also did $TCID_{50}s$ on (New P_3VP_1H and P_3VP_2H

Checked Vero clone 5 - growing fairly well - will sub culture into a 25cm^2 flask on Mon and feed duplicate prior to infection later this week.

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Infected 10 x TC150s (70-80% confluent) each with ± 1ml of Old P_3 Vero and some New P_3 Vero 10am - adsorbed 3hrs → 1pm then fed with 20ml MEM.

Spun down Kinased Oligos → visible pellet - resuspended in 20µl H_2O at ± 500ng µl^{-1}

Set up 1st step in annealing trailer region.

Mixed 2.3µl (± 1.2µg) each of Oligos 205, 203, 202. in x1 NTE with a final vol of 10µl (See page 111).

Step 2 was identical with the addition of Oligos 6 + 8

Step 3 Oligo 4 was added.

Step 4 Oligo 1* was added. * unKinased

Step 5 Oligos 5* and 9

Stored at -10°C prior to ligation/gel purification.

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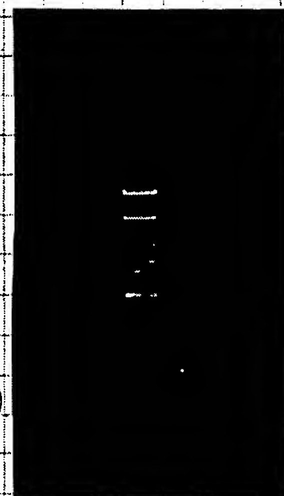
From Page No.

Prepared 1% agarose gel for analysis of trailer annealed products

Run - stained - photographed



Gel shows ≈ 200bp fragment in the approx correct position for the complete ~~leader~~ trailer region.

Will set up a ligation with 50 ng of CAT gene and 10 fold molar excess of Leader and Trailer. Will need 1.2μg of Leader (K^+K^-) and 2.4μg of trailer

≈100ng.

Checked infected Hep-2 cells - not to much c.p.e. visible yet - some syncytia are visible. Will check again this evening.

Checked gradient maker - can probably prepare 4ml 15-55% gradients if use 2.5ml in each chamber.

Ordered SG5 and SG15 (5ml and 15ml) gradient makers.

Will use 4μl from each leader prep K^+ K^- and ppt with ½ of trailer prep and ⅓ of CAT gene prep and then set up ligation.

Mixed 10μl of annealed trailer
4μl of annealed leader
10μl of CAT gene
and ppt^ed all together at -80°C - then set up ligation in 40μl final vol.

4μl x 10 buffer
34μl H_2O
2μl Ligase
40μl

Stored ligation at -10°C O/N prior to gel analysis

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Checked $TCID_{50}s$ - It appears that higher input does give a higher yield of virus but overall yields appear to be a little lower than expected perhaps due to the fact that the Heps were a little over grown when infected. Will make a final read on Fri.

① ② CAT gene ladder

Should set up another ligation with increased amounts of CAT + leader (Keep trailer the same or a little less)

Gel shows products of ligations containing CAT gene, Trailer and leader from RSV

Clearly CAT gene was limiting but some of the correct sized material is present

PUC19 is ≈ 3 times the size of the CAT/RSV construct. Will also use an 2x excess of PUC to favour capture of the appropriate DNA

Would estimate 10-20 ng/4µl for correct species of DNA
in 4µl = 100-200ng
Will use 25-50ng of CAT/RSV and ≈ 200ng -300ng of Vector PUC.
≈ 1/4 of total prep

Also harvested 10x TC150's after 48hrs p.i
(Numerous syncytia were visible.
Went through N.capsid preparation scheme
Spun 15mins at 3000 x g for MEM super. - Kept pellets and spun this super at 27,000 rpm in SW 28 4°C for ~~1.5~~ hrs to pellet 'free' virus - pellets looked good - (small)
Removed cell monolayers with 'Versene' and pelleted on top of 1st super pellets - then took up ~~x~~ pellets in lysis buffer (add small vol. 1st) - Ice 20mins - Dounce 10 strokes ; spun out nuclei etc 1.75K 2mins. - Pelleted N.capsid from super 10K JA20 4°C - Washed (broken up) in lysis buffer - repelleted, Washed with T.E - repelleted - resuspended in 15% K Tartrate (W/W in T.E) - Very difficult to resuspend (use larger Vol. next time + a little D.O.C? SDS?)

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Recorded by David K Clarke

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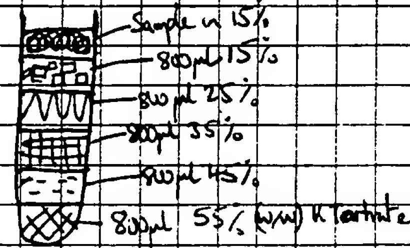
From Page No. 117.

Prepared a total of 6 K^+ Tartrate step-gradients in SW 50.1 tubes



Spun at 29K for 16hrs at 5°C in SW 50.1

Meanwhile resuspended 'Virus' pellet (each in 100µl of NTEx1) on ice (at 4°C) O/N.

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Took off N capsid spin. There was a band of white somewhat flocculent material a little over ½ way down the gradient. Harvested all 6 gradients – diluted x4 in T.E. and pelleted white material in the eppendorf (10 min spin). Poured off super – rinsed with T.E. and then resuspended pellets in 200μl T.E. each – added SDS to 0.2% and x10 Proteinase K left 1.5hr at 37°C (all flocs disappeared).

Then Phenol chloroform extracted (~~this~~ ended up doing x2). Rinsed x2 with Chloroform and ppt^ed with 2 vols of EtOH.

Meanwhile resuspended Virion pellets – did SDS/proteinase K – phenol chloroform extraction and etOH ppt^ed.

Set up ligations

(1) 10μl of K⁺ leader ligation containing some of correct-sized ~~plasmid~~ construct.
5μl of PUC ≈ 250ng.
2μl x10 buffer
2μl H_2O
1μl T_4 ligase
20μl.

(2) 15μl CAT gene
8μl Annealed K⁺ trailer
8μl " K⁺ leader

ppt^ed together – 70% EtOH rinse – dried

then 4μl x10 buffer
34μl H_2O.
2μl ligase.

Put both ligations at 37°C for ≈ 45 mins – then left at R.temp O/N.

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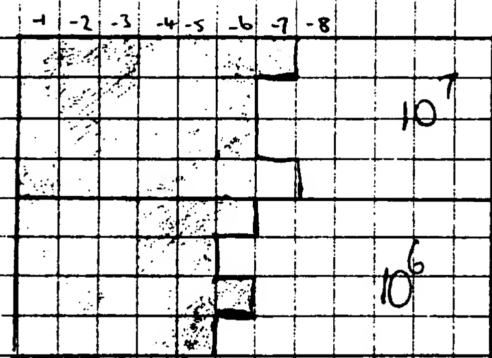
From Page No.__

Prepared 1% agarose gel for analysis of ligation products - will look at 4µl of ligation mixture.

Prepared 200ml of L-Agar Amp+ for Transformation

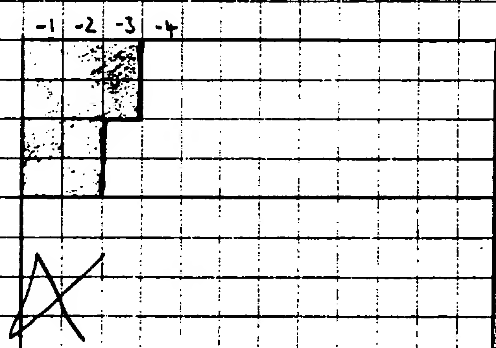


V_3H_2 32°C

New V_3H_1 32°C.

Cells were overgrown - which probably had a significant effect - will repeat soon with more normal Hep-cell monolayers.

Clearly a 10X difference in virus stocks though!



9320 P_1V. 37°C.

B-virus is growing (poorly) on Veros but not at all on Hep's!

9320 P_1 Heps - didn't grow!

Set up transformation using ≈ 50µl competent cells/tube

Eff. control (1µl of control DNA)

CAT DNA con (1µl of purified CAT)

Cut pUC con (1µl of purified stuff)

Ligation Mixture 5µl of 20µl

Ice 1.5 hrs

1min 42°C heat shock

0.9ml SOC. ≈ 1hr at 37°

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Serge W. Kolb (signature)		Recorded by David K Clarke (signature)	

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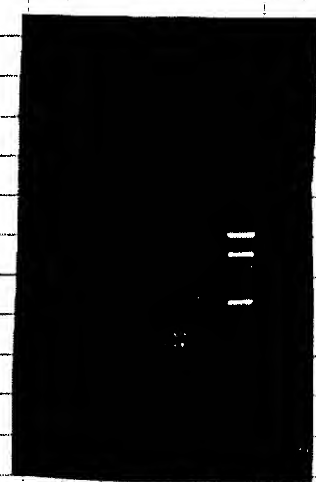
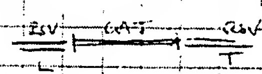


New ligation conditions have pushed more CAT gene into



perhaps 30-40ng in 1/10 of ligation mixture ≡ 300-400ng total

plated transformants on L-Agar Amp (10µg ml^{-1}) containing 80µl of X-gal stock and 8µl of I.P.T.G.

Put 200µl of transformed cells on each plate

Subcultured Vero clone 5 into 2 x 25cm^2 flasks

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From Page No.

Checked transformed cells - lots of white colonies even on the PUC transformed control plate - seems like Blues are secreting β-lactamase allowing satellites to spring up.

CAT DNA alone - Zero colonies - No blues ∴ No whites!
Eff Con - quite a few blues - many whites.
Cat PUC - quite a few blues - many whites
Ligat^n Mixture - Very many Blues and Whites

Will repeat on Monday - using 150µg ml⁻¹ Amp plates and a dilution series of ligation mixture (undil 200µl, 10^{-1} 200µl 10^{-2} 200µl)

Will transform with 4µl of ligation mixture.

The large increase in number of blues in the ligat^n mix Transformation over the CAT PUC, (a similar total amount of PUC in each case) indicates that the ligation did work and regenerated plasmid where only single enzyme cuts had occurred. (either KpnI or HindIII)

It remains to be seen if some double cut plasmid picked up the RSV/CAT DNA.

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From Page No.__

Prepared 200ml L-Agar - poured plates x6 containing 150μg ml⁻¹ Amp

In the meantime infected 10 x TC 225 - each with 1.5ml of 'New' working stock (P_3V ATCC) Adsorbed ≈ 3hrs.

Checked Vero clones - not yet confluent - will infect later this week - also will freeze down one flask

Set up transformations 50μl competent cells 100μl for ligat^n mixture
1μl of either Control 4μl ligation Mix
pUC or Cut PuC.

Left on Ice for ≈ 1hr. 1min 42°C heat shock - 1hr 37°C with 0.9ml SOC.

Infected a 25cm² Vero monolayer with ≈ 1ml of P_1 Vero 'B' 9320 adsorbed ≈ 3hrs then added ≈ 3ml of MEM

Prepared IPTG c̄ 1a Maniatis in sterile H_2O - Filter sterilised - aliquotted and froze at -20°C

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From Page No.

Checked transformations – No breakthrough colonies this time – however there are as many white colonies on the Unligated PUC (cont) as on the initial Ligatn mixture.

Ligation regenerated many blue colonies – indicating that a large proportion of plasmid was cut only once or that many doublets were formed from Head-to-tail ligation of PUC.

Will streak out whites on an amp Meter + Chloramphenicol+ plate. Will also phosphatase PUC and Kinase ligation products – Would not be optimistic about any of the whites since there was an equivalent amount of PUC (and equiv. number of whites) in the Con. and Test transformations.

Prepared Cm+ Agar – poured – dried – plated out Bugs.

Checked infected Veros + Heps – No signs of major c.p.e. yet – will check again at 5-30pm

Phenol Chloroform extracted ligation mixture and then set up Kinase reaction – Pptted PUC DNA with a little glycogen and set up phosphatase reaction.

11am. SAP ran on.

After both reactions, phenol chloroform extracted and ppted

(~~Set up O/N ligation reaction utilising ½ of PUC 19~~)

Will gel purify ligation products before setting up ligation and run ¼ of gel purified material to check % recovery

Streaked out Bacterial transformants on Cm+ and AMP+ plates and grew O/N at 37°C

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Checked bacterial transformants - 3 showed a little growth on Cm$^+$ plates - May not be significant - respread and incubated at 37°c O/N once more. Kept the Amp$^+$ plates at room temp. to allow Blue colour to finally develop.

Went ahead with virus harvest - Clarified supernatant - 20mins at 3K then proceeded with high-speed spin. (c.p.e was well advanced on Hep2 - 48h p.i.) - had to discard some of the super as did only one run on the SW 28

Proceeded to take off cells with Versene - spun down at 3K for 15mins - decanted super and froze cell pellets until tomorrow

Put on Virus spin on refurbished centrifuge - 27Krpm 4°c 1.5hrs

Meanwhile spun down CAT/Leader/Trailer DNA and electroeluted from a 1% Agarose gel.

Checked clones that were showing signs of growth on chloramphenicol plates - they are showing signs of continued growth - will set up 4 mini preps O/N - 3 potential tves and 1 neg

Centrifuge is working fine - took off spin - decanted super, and drained thoroughly - then resuspended O/N in 100 µl of NTE

Used NA45 paper - eluted into 100µl of High Salt NET
Diluted to 0.5M NaCl - added 2µl of glycogen (20µg) and EtOH ppt^{ed}

To Page No.

Witnessed & Understood by me, Sage W. Klob | Date | Invented by | Date

Recorded by David K Clarke

Project No.________

Book No.________ TITLE ________________________

From Page No.__

Removed Cm^R bacteria from incubator - growth looked encouraging

Went through mini-preps to check for CAT/RSV clones

Meanwhile prepared 40% (5cc) - 30% (6cc) - 25% (6cc) - 5% sucrose gradient for isolation of nucleocapsid à la Udem and Cook. Introduced the Dounce homogenizer procedure from the Mike Mink protocol, but otherwise was as published in Udem and Cook.

Set up mini-prep digests on ½ of total DNA in each case

29µl H_2O
5µl x10 BSA
5µl x10 RXN buffer
10µl DNA
1µl Enzyme

50µl 37°C for 1.5 hrs — ppted at -20°C O/N after digestion prior to Hind III digestion.

Set up Isopycnic gradients (x2) for Nucleocapsid in SW50.1 38K for 36hrs

on at 3pm Thurs - off early on Xmas Morning @ 9am

To Page No._____

Witnessed & Understood by me, Gary W. Kobb | Date | Invented by | Date

Recorded by David K. Clarke

Identification of Potential CAT/RSV constructs.

TITLE Preparation of genomic RNA from Virions

Project No.

Book No.

From Page No.

Set up Hind III digestions on mini preps - previously cut with KpnI - to check for CAT/RSV insert - prepared a 1% agarose gel for analysis of digestion products. Also spun down NA45 paper purified CAT/RSV DNA from ligations done earlier in the week - will analysed ¼ of total on the gel.

Meanwhile did proteinase K, SDS digestion of resuspended Virions; then proceeded with Phenol chloroform extraction, and ethanol pptn.

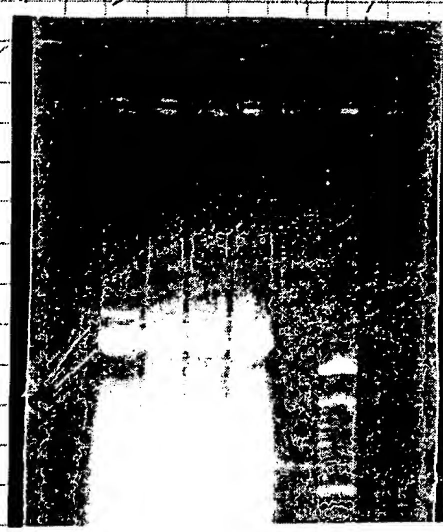
Froze down Vero clone 5 in 90% FCS (heat inactivated) and 10% DMSO

Changed Medium on 'B' 9320 infected Veros, in an attempt to stimulate virus reproduction (3 days p.i.)

Split Hep 2s 1 to 4 (They use 10% FCS)

Split Vero 1 to 10

Mini preps / Eluted / CAT/RSV ladder



Mini preps do not show desired band.

Will proceed with ligation using 5µl of RSV/CAT (≈ 25ng) and 7.5ng of pUC Vector (use ≈ ¼ of phosphatased pUC).

Run religated pUC con

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Witnessed & Understood by me. Date

Invented by Date

Recorded by David K Clarke

000150

Project No.

Book No. TITLE Isopycnic Nucleocapsid gradients off.

From Page No.

Took off CsCl spin (Nucleocapsid) - No N.capsid band was visible; however a distinct RNA pellet was visible at the bottom of the tubes - these pellets were dissolved in 200µl H_2O - phenol chloroform extracted and EtOH ppted. The RNA may be Ribosomal RNA, but there could also be some N.capsid RNA which lost the 'N' protein due to the high salt (CsCl) and SLS. Will try RT-PCR on a representative sample from the various preps.

Checked 'B' 9320 infected Veros - they are showing good signs of c.p.e. (after the medium change at 3 days p.i.)

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kibble		Recorded by David K Clarke	

CAT/RSV DNA Ligation to PUC 19.

Project No.

TITLE: **Book No.**

From Page No.

Checked R9320 infected Veros - c.p.e now well advanced - decided to harvest; however all dry-ice and liquid N_2 was used up so decided to continue incubation at 32°c until tomorrow, when supplies will hopefully be restored.

In the meantime set up ligation of NA45 paper-purified Kinased CAT/RSV and SAP⁺ pUC 19.

25ng x 5µl of CAT/RSV
75ng 1/4 of pUC prep"

20µl final vol
1µl of T_4 DNA ligase 14°C O/N.

Also ran a 'pUC 19 (SAP⁺) ligation control with no insert DNA.

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[signature]

Recorded by David K Clarke

Project No.

Book No.

TITLE Transformation with RSV/CAT/PUC19.
Control RT-PCR Using Neils' RNA + Primers.

From Page No.

Checked B9320 infected Veros – c.p.e. now very extensive – will harvest today as soon as the dry ice arrives.

Veros and Heps need to be passaged today – also need to infect Vero Clone (5) with 'Working stock.'

Set up cDNA synthesis using Neils RNA and primers (3 + 5)

(0.5μg) 5μl RNA + 5μl H_2O, 92°C for 2-3mins to denature –
Snap cooled in ice bath to prevent renaturation
1μl Methyl mercury 14mM → 5mins R.Temp.
5μl 2-Mercaptoethanol 1.85M → 5min R.Temp.
Used 1μl of Neils Primer N° (3) for reverse transcription
Used 1μl of Reverse Transcriptase 1.5hrs at 42°C

Inactivated RTase at 94°C for 5mins.

Then set up PCR RXN – added 2μl each of Neils primers (3)/(5).
and 1μl Taq pol. + Mineral oil

30 cycles
94°C 1min ~~2mins~~
40°C 2mins 2mins
72°C 3mins

Meanwhile did transformation with PUC19 ligated to NA45 purified CAT/RSV (Kinase⁺) SAP⁺
added 5μl of ligation mixture to Invitrogen competent cells
– also did control with ligated PUC19 alone
– also tried transformation with some of Neil's competent DH5αs.

Infected Vero Clone (5) with 0.25ml of 'Working Stock' – adsorbed 2.5hrs at 37°C
Passed Veros and HEPs – will passage again on Wed or Thur am
Harvested P2V B9320 – Stored at -80°C

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by David K Clarke

Relative Transformation efficiencies
Analysis of Neil's PCR RXN.

TITLE: Project No.______ Book No.______

From Page No

Checked Bacterial transformants from yesterday. — Results look encouraging. There are virtually no colonies on the control plates containing KpnI/Hind III cut and SAP'd pUC19 — but there are numerous white colonies on the 'test' plates and some on the Cm+ plates.

Neil's competent cells are actually better than the ones supplied by InVitrogen.

Prepared a 1% Agarose gel for analysis of PCR products — ran 5µL of 100 alongside 1Kb ladder.

Ordered 1gm X-Gal from Amresco.

Checked PCR products from Neil's RNA — No product — indicating that my protocol is not useful in this case. Since Neil did get it to work. Will try this protocol when I get back. / he gets back

Set up 12 mini preps on potential CAT/RSV clones — picked 4 from transformation using Invitrogen Competent cells; 4 from Neil's competent cells, and 4 from those plated on Chloramphenicol — Grew on master plates o/n at 37°C.

To Page No.______

Witnessed & Understood by me, Date
Syr W. Klh

Invented by Date

Recorded by David K. Clarke

Project No.____
Book No.____

TITLE Mini-DNA Preps of Potential CAT/RSV Clones
Virus harvest from Vero Clone 5

From Page No.__

Started mini-preps - CM^A colonies grown in Amp/Cm failed to grow - discarded. Proceeded with DNA extractions for the 8 remaining Amp^R clones.

Checked c.p.e. on Vero clone 5 infected with working stock. c.p.e. is very advanced - will harvest later today and check yield the following week.

Set up KpnI digestions on $1/5^{th}$ of Mini prep DNA (extracted from 1.5ml culture)

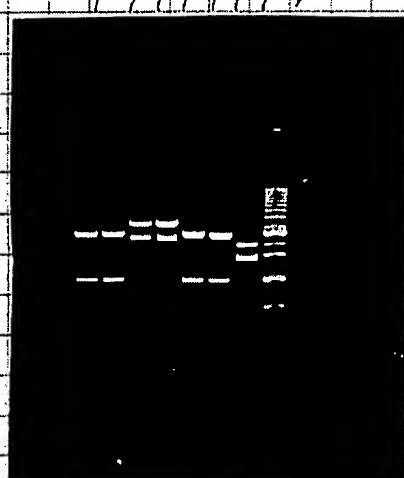
Did rxn in 30µl final vol. for 1hr at 37°C.

Passaged Hep-2s at 1:10 and Veros at 1:20 so they should still be useable on MON.

Phenol chloroform extracted KpnI digests and ethanol ppt^ed - then set up HindIII digests and included RNase A (final vol. 30µl 1hr 37°C)

prepared a 1% Agarose gel and analysed final products.

16 15 14 13 4 3 2 ladder 1



3 4 15 and 16 are it!

1 probably is too.

Harvested Vero Clone 5 cells infected with Working Stock
Stored at -80°C after freeze thaw (prior to crude spin)

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Witnessed & Understood by me, Serge W. Kibble | Date

Invented by | Date

Recorded by David K Clarke

Project No.
TITLE: CATCHING UP ON JOURNALS! Book No.

From Page No.

Checked growth of bacteria of CM^{+} plates - No go - if the CAT gene is present (and it appears that it is) then it is not being expressed.

Copied a bunch of papers from Virus journals etc. for reading purposes.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Klebe		Recorded by David K Clarke	

Project No.

Book No.

TITLE Checking CAT/RSV CONSTRUCT By HgA I digestion.

From Page No.

Took a look at passaged Vero/Hep-2 (from last week) - They appear to be okay, but will probably wait and use the new cells.

Digested plasmids 3, 4, 15, 16, with HgA I which should linearise the plasmids if they contain the correct insert.

3μl x10 buffer
10μl plasmid
1μl Enz
16μl H_2O → + 1μl RNase A
30μl

1-2hr at 37°C

Meanwhile prepared a 1% Agarose gel for analysis of digestion products

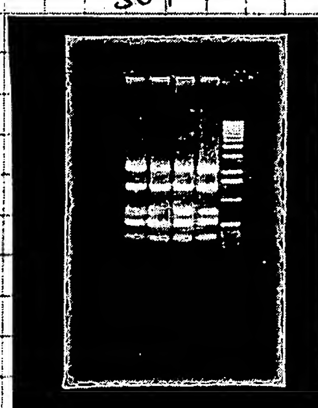
Ordered Perkin Elmer RT-PCR Kit.

There are 5 DNA bands when the CAT/RSV construct is digested

1420* = Key band containing CAT/RSV DNA
747
586 } probably migrate together
560 }
307

Set up O/N mini cultures of 4 and 16 (10μl Amp 50μg μl⁻¹)

Autoclaved



All requisite bands appear to be present in the correct position

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

000157

Project No.
TITLE: Storage of CAT/RSV clones.
Book No.

From Page No.

Took off O/N cultures of CAT/RSV clones 4 and 16.
Set up glycerol stocks of each culture.
150µl of sterile glycerol
850µl of bacterial culture.
Vortexed briefly and stored at -80°C.

Prepared 2 x 500ml L-b broths - autoclaved

Prepared a stock of Amp 50mg ml⁻¹ (filter sterilised - aliquotted and stored at -20°C)

Set up O/N plasmid cultures - added 500µl of Amp 50mg ml⁻¹ to each flask + 500µl each O/N mini prep culture

To Page No.

Witnessed & Understood by me, Date
Invented by Date
Recorded by David K Clarke

Project No.____
Book No.____

TITLE Lage Scale Plasmid Preps of CAT/RSV

From Page No.__

Began plasmid preps – Spun bugs down 3.5K 30mins JA-14 rotor

Resuspended pellets in 10ml P1 (Qiagen series of solutions) – then added 10ml P2 (freshly made 0.2M NaOH 1% SDS) and incubated at R. Temp for 5-10mins. Then added 10ml P3 – mixed thoroughly/gently and incubated on ice for 20mins. – Spun in JA-20 for 30mins at 18K.

Ppted the super with 0.7 vols Isopropanol and immediately spun at 10K for 45mins in the JA-20. – Poured off super and rinsed pellet with 70% EtOH (10ml) – repelleted 10mins.

Air dried pellet 10mins – then redissolved in 400µl T.E buffer – retained 1µl for gel analysis and EtOH ppted the remainder – there was quite a bit of chromosomal DNA present (fished out with an inoculatn loop).

Stored ppt in freezer in lab.

Ordered 293 cells from ATCC (see opposite page)

Ordered T7 Polymerase (5000 u) from Promega

Denatured DNA from NaOH treatment

There appears to be ≈ 500ng of plasmid in 1/400th of total

∴ total ≡ 200000 ≡ 200µg of each plasmid in 1ml EtOH.

≡ 200ngµl⁻¹ in EtOH.

To Page No.____

Witnessed & Understood by me. Sez W. Khl

Date —

Invented by

Date

Recorded by David K Clarke

‡00B

293 cells

137

.HEADLINE
ATCC CRL-1573 293 (Transformed primary embryonal kidney, human)
.TEXT
Passage Frozen: 31. Current medium for propagation: Eagle's MEM with Earle's BSS, 90%; heat-inactivated horse serum, 10%. Additional Information: The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and contain and express the transforming genes of Ad 5. Handle as potentially biohazardous material under at least Biosafety Level 2 containment. The line has been used in the isolation of transformation defective, host-range mutants of Ad 5, and is excellent for titrating human adenoviruses. This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells. References: J. Gen. Virol. 36: 59-72, 1977; Virology 77: 319-329, 1977; ibid., 86: 10-21, 1978. Submitted by: F.L. Graham, McMaster University, Hamilton, Ontario, Canada. Price Code: J

Project No.______
Book No.______

TITLE Checking large Scale CAT/RSV plasmid Preps
Control RT-PCR with Perkin Elmer Kit.

From Page No. ______

Spun down 50µl of each CAT/RSV plasmid (≈ 10µg) – EtOH rinsed
Dried and resuspended in 10µl H_2O (1µg µl^{-1}) Then set up KpnI digestion
on 2µl

2µl DNA
5µl x10 buff
5µl x 10 BSA
36µl H_2O

Phenol Chloroform extracted and EtOH ppted.

Proceeded by setting up HindIII digestion

3µl x10 buff
26µl H_2O
1µl Enzyme
30µl 1.5hrs 37°C

Meanwhile prepared 1% Agarose gel and run digests

Perkin Elmer Cetus RT-PCR Kit arrived – decided to set up control run – using
Their control RNA + primers.

Set up reverse transcription of control RNA according to their protocol – after
inactivation of RT at 99°C for 5mins proceeded with PCR portion of control. (see protocol for details).

(Did not carry out initial 2mins at 95°C)

Ordered
DH5 α competent cells from BRL.

little bit of one-cut plasmid

little bit of uncut plasmid

Gel shows that the large scale plasmid preps of (4) (16) CAT/RSV are okay and giving the expected products following KpnI/HindIII digestions.

To Page No. ______

Witnessed & Understood by me, Sag W. Klub | Date | Invented by | Date

Recorded by David K Clarke.

Analysis of P.Elmer Control PCR RXNs

TITLE Preparation of RSV/CAT Constructs for In Vitro Transcription

Project No.

Book No.

From Page No.

Set up HgaI digestion on 8μg each of RSV/CAT ④ ⑯

43μl H_2O/DNA
5μl x10 buffer
2μl Enz.
50μl

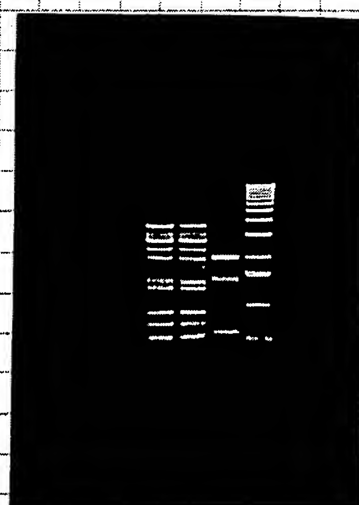
2hrs 37°C on at 9-30am
off 11-30am

Prepared Gel for analysis of PCR product (from O/N RXN) and digestⁿ products.

Phenol Chloroform extracted HgAI digests and EtOH ppted.

Set up Spectrophotometer (arrived in from Baxter)

It also appears that the control RT-PCR RXN did not work. Will set up again. This time used gloves! and some of Neil's RNA and one Rxn with Life Science R.T. also included a 60°C annealing step.



Gel shows incomplete HgAI digestion. Will spin down and redigest for 2hrs in ~~60~~ 50μl using ~~3~~ 2μl of enzyme - 2 hrs.

Will analyse on a gel tomorrow along with PCR products.

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

Project No.
Book No.
TITLE Checking Results of CAT/RSV (with HgA I)
Checking P.Elmer Control RT-PCR RXNS

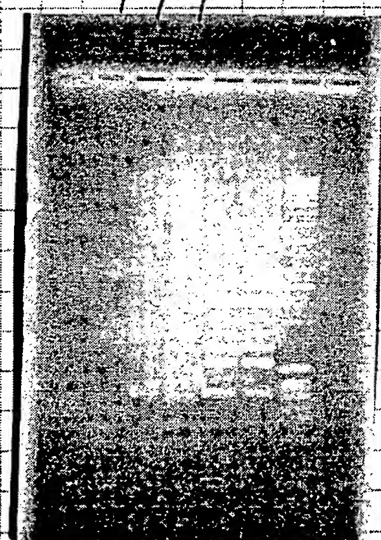
From Page No.

Prepared a 1% agarose gel for analysis of O/N PCR reactions

- (1) Using Perkin Elmer RT-ase
- (2) Using Life Science RT-ase
- (3) Using Neil's Control RNA (from his P.Elmer Kit)

Also checked redigests of CAT/RSV (4) (16) (with HgA I)

Life Science
My RNA
Neils Con RNA



At least 2 of the PCR RXNs have worked
10-20 ng of DNA
≡ 100-500 ng of total product.

Life Science Rev. T-ase did not give a product under these conditions

Looks as though (4) (16) have cut further with HgA I - but still not completely - will order a fresh batch of Enzyme

The enz. conc. is actually v. low - explains why cutting is incomplete

PCR CONDITIONS WERE 2' 95°C ⇒ Ice prior to Taq addition.
Taq added (½ µl) overlaid with Min. oil

94°C 1'
1 min
60°C 2'
1 min
72°C 3'
1 min

Will try Dave's Neil's RNA Next

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by

Recutting CAT/RSV for 3rd Time!

TITLE Setting up More RT/PCR Controls.

Project No.

Book No.

From Page No.

Recut RSV/CAT ④/⑪ – boosted vol to 80µl and added 4µl more of HgAI – incubated at 37°C for 2hrs until – then analysed 4µl on a 1% Agarose gel – Phenol Chloroform extracted and EtOH ppt^ed

Infected Vero cells (25cm²) with 10^{-1} and Undil B9320 – 0.25ml of each. Also infected 1x 25cm² with $V_3 H_2$ (32°C grown) – 0.25ml

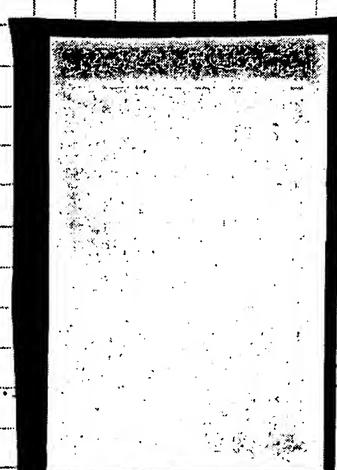
(Will grow Hep-virus at 32°C ; Vero virus at 37°C)

Set up reverse transcription on X31 RNA (supplied by Dave Stec).

Stick to P. Elmer Protocol in general but with one difference:

Added 3µl 555 – Daves Primer to 5µl of RNA (≈ 4µg)
Heated to 90°C for 1.5mins then snap cooled on ice – then added 3µl of this mixture to the RXN mixture and incubated at 43°C for 1hr 2:30pm - 3:30pm

Good all the HgAI fragments are as they should be



200ng total in 4µl x20
= 4,000 ≡ 4µg of cut plasmid

P.T.O

To Page No.

Witnessed & Understood by me. Serge W. Klele (?)

Date

Invented by

Date

Recorded by David K Clarke

Project No.______

Book No.______ TITLE ______________________

From Page No. 141

After Reverse Transcription – heat denatured RT-ase at 80-90°C for ≈ 5mins, Then added the PCR ingredients including 2µl of 556

After mixing all ingredients did another 95°C treatment for a few mins to fully denature the DNA/RNA hybrids → Ice + 0.6µl Taq – overlaid with mineral oil → 94°C 1min

1min

↓

37°C 2mins

2mins

↓

72°C 3mins

1min

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Khl[illegible]			
		Recorded by David K Clarke	

Checking PCR of Fin Rxn

TITLE Harvest of V_3H_3 32°C Virus.

Project No.

Book No.

From Page No.

Prepared 1% Agarose gel, and loaded 1/10 (10µl) of O/N PCR reaction to see if 1.4Kb 'Fin' DNA was produced.

In the meantime checked c.p.e. on infected Vero/Hep-2 cells

Veros infected with V_2 B9320 were not showing much c.p.e. - will check again this evening.

The Hep-2s infected with V_3H_2 were blown away! - Harvested and froze down as V_3H_3 (32°C) Will titrate on thurs

Good - the PCR RXN has worked giving a total of ≈ 1µg of product.

Oligos 155
154
are the ones to be used to test the RT/PCR for RSV - set annealing at 37°C

Dave's Oligos used were SS5 and SS6. They were approx. 30-mers with a M.P of 74°C. AT rich oligos.

Should probably set annealing temp for RSV work at 20-30° below M.P (or 37°C) whichever is highest. also 2' between 94°C and annealing temp and 2' between annealing and 72°C

Dil 155 by 1/3

Dil 154 by 1/6. Use 154 as cDNA primer

To Page No.

Witnessed & Understood by me, Serge W. Kobe (signature) | Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Optimization of Flu ~~RNA~~ RT/PCR Using CH_3Hg etc.

From Page No.

Checked c.p.e on Vero cells - Vndil shows good c.p.e - Strangely it had leaked (perhaps by capillary action). Replaced [illegible] of growth medium and continued incubation - will harvest this evening or tomorrow morning.

Set up a 2nd series of RT-PCR reactions

1. Based on Methyl Mercury denaturation
2. Based on no heat denaturation of RNA/cDNA (Just the usual 1' at 94°C during thermal cycling).

1. Heat denatured RNA 90°C 2' → Ice
   added 1μl of 14mM Methyl Mercury → 5mins RT
   added 1μl of 1.85M β-Mercap → 5mins RT.
   added 3μl of ~~[illegible]~~ SS5 primer and then added 4μl total to Reverse T-ase mixture - 1μl of R-T-ase → 42°C for 1.5hrs.

2. Heat denatured RNA + 3μl primer 90°C 2' → Ice.
   added 3μl of this mixture to R-Tase mixture - 1μl RT-ase → 42°C for 1.5hrs

To Page No.

Witnessed & Understood by me, Serge W. Kholo [signature] | Date | Invented by | Date

Recorded by David K Clarke [signature]

Project No.
TITLE: Continued from 144
Book No.

From Page No. 144

Set up PCR on 1 after 5mins at 90°C to inactivate reverse transcriptase.
Used ≈ 1µl Taq. 1.5µl of 2nd primer (SS6) + additional ½µl of SS5

Phenol chloroform extracted the 'No heat' treatment [2] - EtOH ppt^ed with 2µl of glycogen - will set up PCR with these products tomorrow.

Meanwhile harvested B9320 infected Veros → V_3 B9320 dl/undil.
Froze away at -80°C - will use to infect Veros tomorrow and do TCID50s.

To Page No.

Witnessed & Understood by me, Alyce W. Kelble | Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE TCID50s of B9320/Clone 5 Vero/V3H3

Analysis of PCR Products incorporating Methyl Mercury.

From Page No.

Prepared 1% Agarose gel for analysis of PCR products.

LHS. loaded 1Kb ladder / X31 / Methy Merc. / (undil).

Infected Vero Cells with P3 Vero B9320 → 10^{-1} / undil

Carried out TCID50 on – P3 Vero B9320 10^{-1} undil.

– Vero Clone 5 progeny (infected with Working stock).

– V3H3

X31 Met. Merc + heat denaturation

X31 heat denaturation only

Products are essentially the same with or without methyl mercury – however this does not preclude that Meth Merc. may be useful for V. long cDNA production.

Should try 1/100 dilution of this RNA = 2ng of template = 2×10^9 molecules

Set up PCR reaction with 'No Heat' sample.

optimise Annealing temp. + elongation time heat denaturat'? Solvents?

Resuspended cDNA pellet in

70.4 µl H_2O
10µl x10 buff.
8µl $MgCl_2$
2µl each dNTP
1.5µl Each primer
0.6µl Taq.

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by David K Clarke.

B000169

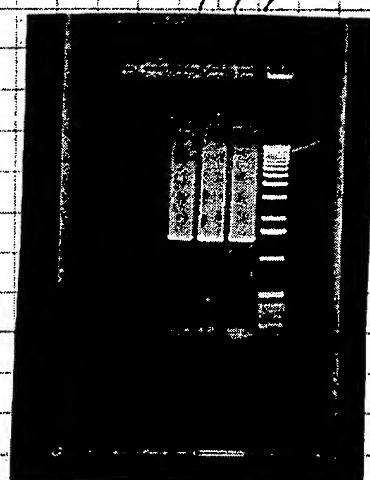
Analysis of Non-denatured cDNA/RNA as PCR Template.

TITLE Reverse Transcription/PCR on Potential RSV genome. **Project No.**______ **Book No.**______

From Page No.__

Prepared 1% Agarose gel for analysis of O/N PCR Reaction (No heat used to initially denature RNA/cDNA hybrid). Will run samples from 'X31' and 'Methyl Merc' RXNs as controls.

Checked c.p.e on B9320 infected cells – there are already some c.p.e will probably be able to harvest Tomorrow.



It appears that all of the conditions used were equally successful in producing a PCR product – will probably go with the Methyl mercury approach and a short 1' 'burn' right before adding Taq.

Set up 4 Reverse Transcription reactions utilising the various

Nucleocapsid K⁺Tartrate / CsCl.

Virion. 12-14 / 12-27 preparations

Suspended RNA in 10µl H_2O (DEPC treated) and 5µl of Oligo 154 (100ngµl^{-1}) – Heated to 90°C for 1' → Ice – added 1µl Methyl Mercury, followed by 1µl of 2-mercaptoethanol – Added 4µl of this mixture to the Reverse transcription mixture and 1µl RTase → 42°C for 1 hr

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Witnessed & Understood by me,	Date	Invented by	Date
Syze W. Kible		Recorded by David K Clarke	

Project No._______

Book No._______ TITLE ____________________

From Page No.__

See - reference binder #22A

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

Project No.

TITLE Book No.

From Page No.

Checked Infected Vero cells (B9320) — They have been 'blown away' at 10^{-1} and 'Undil' — Harvested and froze down as B9320 P_4 Vero 10^{-1}/Undil'

Checked 293 Cells — Many had settled down and were growing okay; Poured off the supernatant containing dead floating cells and re-fed with 5ml growth medium.

Prepared a 1% agarose gel and analysed 10µl samples of O/N PCR RXN

Vinun 12-24-93.
CsCL
Vinun 12-8-93
K^+ Tart

Fantastic — all the various RSV genome preps have given me a RT-PCR product of 550bp

To Page No.

Witnessed & Understood by me, Sige W. Kble

Date

Invented by

Date

Recorded by David K Clarke.

Project No.______
Book No.______ TITLE Preparation of New Virus Working Stock (P_3VP_1H).

From Page No.__

Checked $TCID_{50}s$ - B9320 Virus appears to have grown v. well; about $10^6 ml^{-1}$

Vero clone 5 is also producing 10^{-5}-10^{-6} ml^{-1} which is quite a good titer.

V_3H_3 titer is much lower than expected - perhaps because virus sat too long at 37°C when all the cells were wiped out (before harvest) or perhaps the virus is adapting to Hep-2s so well that they are not growing well on Vero (indicator cells!)

Will make final $TCID_{50}$ readings tomorrow

Checked 293 cells - they are growing fairly well (without Horse serum) ≈ 70% confl.

Ordered Lipofectace Reagent (1ml) from BRL. 2nd Day delivery.

Infected Hep-2 (TC 150s) with P_3Vero (working stock) - used 1ml/flask

Infected Veros (TC 25s) with ["Undil" P_4V B9320] 10^{-1}/Undil.

Adsorbed ≈ 3hrs at 37°C.

Will need oligo for making 1st RSV L-clone (Have cDNA primer)

5' ACG/AGA/AAA/AAA/GTG/TCA/A 19-mer

Mp. ≈ 50°C

This clone will be ≈ 3Kb (2471) if it works out.

To Page No._____

Witnessed & Understood by me, Serge W. [signature] Date

Invented by Date

Recorded by David K Clarke.

Project No.

TITLE: Book No.

From Page No.

Will use some of Virus RNA/Primer 154 (already at -80°C) and some fresh EtOH ppt from K+ tartrate

To Page No.

Witnessed & Understood by me, Date Invented by Date

[signature]

Recorded by [signature]

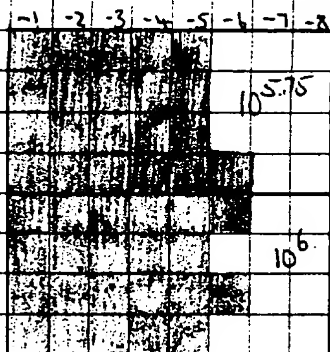
Project No. ______

TCID$_{50s}$.

Book No. ______ TITLE In Vitro transcription of CAT/RSV.

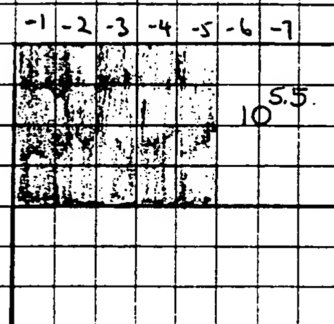
From Page No.

Checked infected vero cells - no signs of c.p.e yet - will check again later this afternoon. Hep-2s are showing a few syncytia - should be okay by tomorrow.

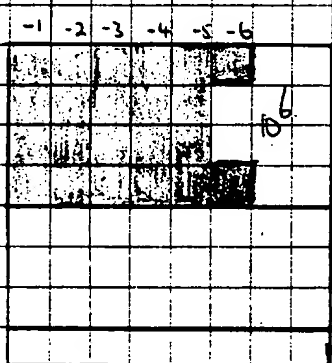


89320
P_3 10^{-1} inf.

Undil (inf.)



V_3H_3
undil.



Vero Clone 15
P_1V (infected with working stock)

293 cells are looking pretty good ≈ 80-90% confluent.

Set up In Vitro transcription of CAT/RSV (HgA I) digested 4 and 16

4µl x5 Rxn buffer
2µl 100mM DTT
½µl RNasin
4µl rNTPs
1µl DNA Template (≈1µg)
7.5µl H_2O.

20µl final Vol

off at 4-20 pm

+ 1µl of T_7 Pol.

To Page No. ______

Witnessed & Understood by me, Serge W. Kibble [signature] | Date | Invented by | Date

Recorded by David K Clarke

TITLE Gel analysis of IVT CAT/RSV RNA. Virus Harvest. P_3 V P_1 H-2.

Project No.________ Book No.________

From Page No.__

Checked infected Veros – they are not showing v. advanced c.p.e – perhaps lost titer in previous infection (since 10^{-1} is not showing good c.p.e either, it probably is not due to DI buildup. Will leave until tomorrow to harvest. Will wait to titrate P_4V and P_5-V B9320 before deciding on any further infections.

Checked HEP-2s – There are quite a few syncytia – will harvest later today

Prepared 1% MOPS Agarose gel with Formaldehyde

5ml x10 Mops
37ml H_2O
8 ml Formaldehyde

Took 4µl aliquots from IVTs 4/16 – added to 10µl deion. formamide – 10µl X1 mops gel buffer – added 1µl of 1mg ml^{-1} EtBr – heated to 65°C for 5mins – added 2µl of BPB and loaded on gel with 1.5µl of RNA markers.

Checked 293 cells – They look reasonable, but will require a couple more days before they are fully confluent.

Harvested V_3H_1 virus (48h.p.i.)
There were quite a few syncytia
Stored frozen as V_3H_1 32°C

Also shifted Veros infected with B9320 (Until) to 32°C (left 10^{-1} at 37°C)

Seems to be the correct size of transcript for CAT/RSV.

1,892 marker.
872 marker.

To Page No.____

Witnessed & Understood by me, Sege W. Kh[illegible] | Date | Invented by | Date
Recorded by David K Clarke

Project No.______
Book No.______ TITLE More In Vitro transcription of CAT/RSV. Gel Analysis of Transcripts

From Page No.__

Checked 293 Cells - they are not growing quickly - will take at least until tomorrow to achieve 80-90% confluence

Set up In Vitro Transcription of CAT/RSV

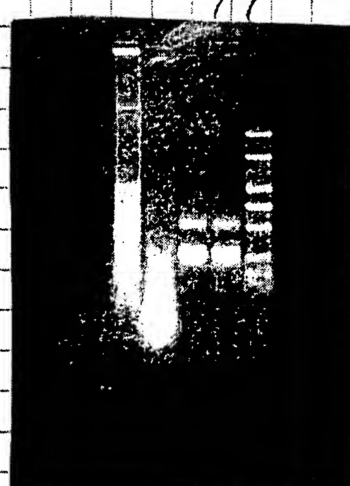
8µl x5 buffer
4µl 100mM DTT.
1µl RNasin
8µl rNTPs 2.5mM each — 1hr 37°C
2µl DNA Template
15µl H_2O.
2µl T_7 Pol
40µl

Meanwhile prepared 1% Mops/Agarose Gel.

Loaded Gel LHS/ RNA Marker 1.5µl / IVT 4 4µl / IVT 16 4µl / Virion Prep 4µl / K.Tert N.capsid Prep 4µl /

Harvested V_5 B9320 10^{-1}/undil - will do TCID50s this afternoon.

IVT 16
IVT 4



15K?
9493
6625 } 3263
} 2314

In Vitro transcription looks v. good.
It seems there is 0.5-1µg of RNA transcript in 4µl of IVT mix.
≈ 1/1500
700ng/band
Will use 10µl for Transfection

Gave Kim Vero Clone 5 for growth and testing.

To Page No.____

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

From Page No. 154

Carried out TCID$_{50s}$ on P_4 Vero 89320 } 10^{-1}/undil.
P_5 Vero 89320 }
V_3H_1 (Stock) 32°C.

Diluted Oligo (RSV 5' to Pml) to 100ng µl^{-1} (working stock).

Restreaked CAT/RSV clone bank → 37°C O/N

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Preparation for CAT Assay and Transfection of 293 cells

From Page No.

Checked 293 cells - they look pretty good - some parts of the monolayer are a little sparse, but others are showing higher density

Restreaked Clones are not growing V. vigorously - will restreak again on a fresh Amp+ plate, and leave at 37°C o/n again to boost growth.

Went over CAT Assay with Pamuk.

Protocol for Transfection of CAT/RSV Transcripts

1) Infect ≈100 confluent 293 cells (in 6 well plate) with RSV with as high a M.O.I. as possible - Consider 10^6 293 cells per 6 well plate - will use ½ml of highest titer virus stock (≈10^7 ml^{-1}) to give an M.O.I. of ≈ 5/cell → 37°C

2) ≈ 1hr after putting on virus remove inoculum - rinse cells x2 with Opti-MEM reduced serum medium

3) Add transfection mixture (1ml) and incubate cells for 3hrs → 5hrs at 37°C (Can leave o/n like this)

4) ~~Then add 1ml of DMEM containing % serum~~ (not really necessary)

5) Retain some of the Supernatant (add a little $MgSO_4$ and Hepes) for possible passage

Set up reverse transcription/PCR as before - extended polymerization cycle to 5mins and used RSV 5' to Pol primer.

To Page No.

Witnessed & Understood by me, Serge W. Klah

Date

Invented by

Date

Recorded by David K Clarke

Project No.
Book No.

TITLE Analysis of RT/PCR products

From Page No.

Prepared 1% gel for analysis of RT/PCR products

Loaded 10µl of 'Virion', 'Nucleocapsid - K⁺ tart' and 1µl of 1KB ladder

LHS / 1KB ld / Virion / N cap

Stored Bacterial clones at 4°C - the plates are too thin to support vigorous growth - will restreak again early next week, on my own plates.

NC V

Results look v. encouraging. It now seems possible that long PCR products are possible - perhaps DMSO may increase yield. - Could also use more template.

Will electro elute purify and clone

Future experiments

1) Test Monoclonal antibody efficiency of neutralisation of B9320. (Plaque / $TCID_{50}$ reduction titre)

2) Transfect in K^+ tartrate gradient genomic RNA.

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by

Project No.______

Book No.______

TITLE TCID50 reading. Infection of Hep-2s. for N. capsid. Transfection of 293s. with CAT/RSV RNA.

From Page No.__

Checked TCID50s — V_3H_1 show a titer of ≈ $10^{6.5}$ — good — this will be an Ideal stock for infecting Heps for N. capsid.

TCID50 of V_4/V_5 B9320 Dil/undil — indicates that should use 10^{-1}/undil of "10^{-1}" V_5-B9320 for next infection — will show final read out tomorrow.

293 cells look pretty good.

Infected Hep-2 cells ($225cm^2$ monolayers) each with 1.5ml of V_3H_1 (from 1-19-94) — adsorbed 3hrs at 37°c then fed with 20ml of MEM and incubated 48hrs at 32°c. 10am — 1pm.

Ordered Oligos Bam HI to Pml and Pml to Bam HI.

mp 50°c 18mer	Bam to Pml	5' GAC / AAA / ATG / GAT / CCC / ATT
mp 50°c 17mer	Pml to Bam	5' CTC / ACC / ACG / TGT / TAA / AC

Used V_3H_2 32°C to infect 293 cells (10^4 pfu ml^{-1}) ≈ 0.5ml / 6well plate = m.o.i. of ~~5-15~~/cell
~~(rinsed monolayers 1st with PBS — should use prewarmed medium).~~

adsorbed for 1hr at 37°C with occasional gentle shaking

Meanwhile prepared Optimem / Transfectace mixture 90µl : 12µl resp.

Then prepared RNA/optimem mixture 10µl : 90µl resp.

To Page No.____

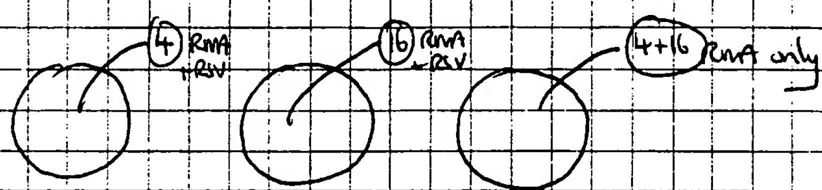
Witnessed & Understood by me, Serge W. Kelly | Date

Invented by | Date

Recorded by David K Clarke

From Page No. 158

Then at a suitable time point mixed RNA/optimem with Transfectam/optimem. Let sit at room temp. for 15mins, then diluted with 0.8ml of Optimem and added to monolayers which had been pre-rinsed x2 with Optimem – left o/n at 37°C.



To Page No.____

Project No._______
Book No._______ TITLE ________________

From Page No.__

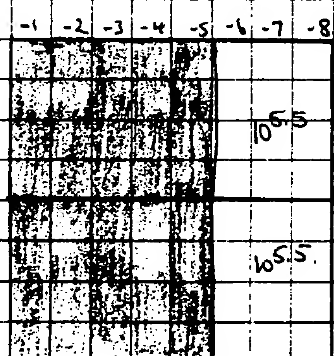
Checked c.p.e. on infected Heps / 293 cells —

Did final reading on TCID50s

293 cells look v. sick - especially those which have been infected with RSV - will harvest this afternoon.

V_3H_1 32°C

Good yield — ~~will~~ used this stock to prepare another batch of nucleocapsid i.e. can use as working stock.

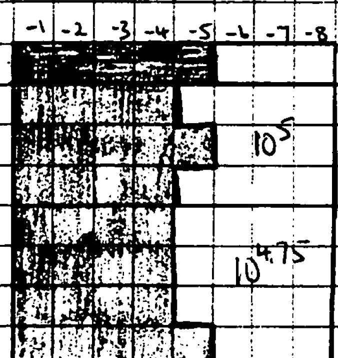


V_5 B9320

10^{-1}

Undil.

B virus is growing fairly well - will use "10^{-1}" stock for P_6 passage to see if can boost titer any more - perhaps can move into Hep's.



V_4 B9320

10^{-1}

Undil

Titers may be affected by virus ½ life at 37°C i.e. not good to leave once c.p.e. is v. advanced - may have happened here.

To Page No.____

Witnessed & Understood by me, Date Invented by Date

Serge W. Kubala

Recorded by David K Clarke

Project No.
TITLE: Book No.

From Page No.

Will order 1 small Hep + 2 small Veros for next Mon.

Need to set up Plaque reduction assay B9320/A2 using Monoclonal antibody in preparation for genome rescue using B9320 as helper.

3pm initiated harvest of CAT Extract.

1. Aspirated off supernatants → froze in EtOH/Dry ice
2. Rinsed x2 with 2ml of cold PBS.
3. Added 1ml of x1 TEM and resuspended cells; by scraping etc.
4. Spun at 3K rpm for 1min at 4°C in eppendorf
5. Resuspended pellets in 100μl of 0.25M Tris pH 7.5 ice-cold
6. went through x3 freeze thaw cycles - Dry ice/EtOH ↔ 37°C
7. Spun down debris 5mins, 14K eppendorf 4°C - collected super and froze rapidly in EtOH/dry ice.

Checked c.p.e. on Heps - already starting to develop fairly well.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kubala		Recorded by David K Clarke	

Project No.______
Book No.______ TITLE RSV Nucleocapsid prep. CAT Assay of transfected 293 cells.

From Page No.__

Prepared 100ml of L-Agar - added Amp and poured plates for restreaking of Clone banks.

Initiated Nucleocapsid prep in 10 x TC 225s which were showing advanced c.p.e. (fusion).

Followed Extraction protocol carefully - used ≈10ml of lysis buffer per 5 x TC 225s.

prepare 6 K^+ tartrate gradients 55% (w/w) → 15% (w/w)

Used 800µl of each conc. but only 240µl of 15% since nucleocapsid was resuspended in 15% K^+ tartrate.

Meanwhile restreaked CAT/RSV clones on Amp^+ L-agar plates

Also set up CAT assays using 20µl of Transfected cell extract
2µl ^{14}C-cloramphenicol (ideally 200µCi ml^{-1}).
20µl 40mM Acetyl CoA
in Tris-Cl pH 7.5.
See protocol - Run for 3hrs at 37°C → -70°C O/N

Put on K^+ tartrate spin at 6pm - should be off at 10am

Diluted Oligos Pml to Bam and Bam to Pml to 100ng $µl^{-1}$
Stored at -20°C - stored.

Ordered Trial Oligo from "Oligos etc."

5'-GGG/GCA/AAT/ACA/AAG/ATG/GCT/CTT/AGC/
AAA/GTC/AAG/TTG/AAT/GAT/ACA/CT-

To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	—	Recorded by David K Clarke	

TITLE: Running of CAT TLC. Harvesting Nucleocapsid from gradient. + further purification of virus Genome

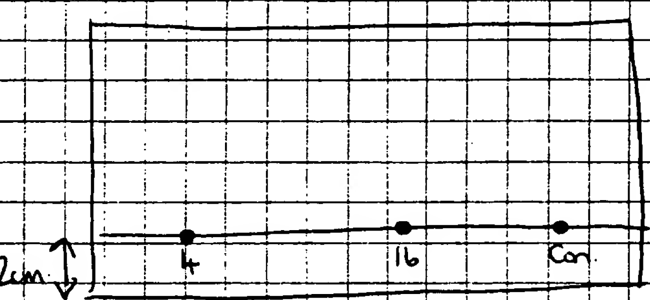
Project No.

Book No.

From Page No.

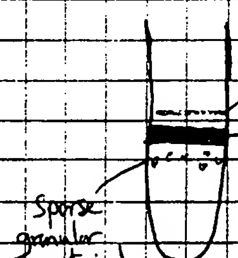
Ethyl acetate extracted CAT RXNS - used 1ml with 3 x 10 sec pulses of vortexing for each - collected ethyl acetate and speed vac'ed down + then resuspended in 30µl ethyl acetate prior to dotting on TLC

Meanwhile equilibrated chromatography tank with 190ml Chloroform 10ml of Methanol. Dotted on resuspended samples - using a capillary tube.



Let TLC go for 2hrs (2pm off)

Realised that I screwed up Ncapsid prep (forgot to do nucleus spin)



Harvested different fractions and tried to pellet Ncapsid band, by dilution into SW 50.1 tubes and spinning at 36K for 1hr 5°C

Also tried SDS proteinase K digestion of thick band of material - seemed v. resistant to digestion

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by [signature]

Project No.

Book No. TITLE

From Page No. 163

Resuspended potential nucleocapsid in H_2O and SDS/proteinase K treated for 1hr at 37°C – then phenol chloroform extracted and EtOH ppt'd.

Somehow didn't look quite right.

Completed TLC, and put away for autoradiography.

To Page No.

Witnessed & Understood by me, Serge W. Kobele | Date | Invented by | Date

Recorded by David K Clarke

Checking N.capsid prep for presence of Genomic RNA.

TITLE

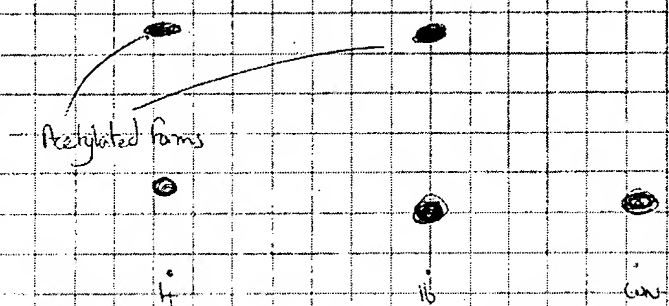
Project No.

Book No.

From Page No.

Developed CAT/RSV rescue autorad — Safe light was not safe!!

However can see that the rescue worked — re-exposed autorad — will develop tomorrow.



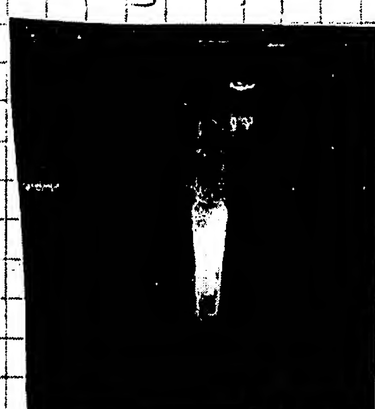
Ran out 3Kb PCR products Virion/Nucleocapsid on a 1% agarose — purified onto NA45 paper and eluted into high salt buffer — Phenol chloroform extracted diluted X2 with H_2O and EtOH ppt^{ed} with 10µg glycogen.

Proceeded to set up O/N ligation with T/A cloning vector (Assume 45ng for Virus PCR product)

2µl x10 buffer	2µl x10 buffer	
2µl Vector	2µl Vector	450ng ÷ 2 = 225ng for Ncapsid PCR product
DNA insert 50ng (say 5µl of 10)	DNA insert in 15µl H_2O (All of 3KB Virion) 12°C /ON.	
13µl H_2O		
1µl T4 DNA ligase	1µl T4 DNA-ligase	

Also Ran a MOPS/Agarose gel to analyse potential Nucleocapsid preps

Neither prep looks promising.



Stained material does not seem to be in the correct position for genomic RNA

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Ligation of RT/PCR Products (3Kb frag.) to T/A Vector.

From Page No.

Set up ligations of 3Kb RT/PCR product - electro-elute purified on NA45 paper from yesterday

3Kb from Ncap	3Kb from Virion	Control
2µl x10 lig^n buffer	"	"
2µl Vector (50ng total)	"	"
5µl Insert DNA (50ng)	15µl insert (50ng)	15µl H_2O
10µl H_2O		
1µl T_4 Ligase.	1µl T_4 lig	1µl T_4 lig.
20µl	"	"

Stored remaining 15µl at -10°C

Used total amount due to low yield.

Left O/N at 12°C

Redeveloped CAT/RSV Rescue TLC - looks v. good Both (4) and (11) constructs are giving positives - CON is Neg.

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

Project No.
TITLE: Bacterial Transformations with Potential JK viral sequence. Book No.

From Page No.

Carried out Bacterial transformations — used 3 set of different competent cells.

1) Neil's
2) Invitrogen T/A system.
3) BRL Transfinity

Added 2µl of each ligation reaction (Cn, N.cap, Virion) to the BRL Transfinity; used 5µl from each ligation mixture for the other Bacterial cells.

Heat shocked BRL/Neil's cells for 1min at 42°C
T/A cells 30secs at 42°C

Added SOC and incubated at 37°C for 1hr — then plated out 200µl from each mixture → O/N 37°C

To Page No.

Witnessed & Understood by me, Serge W. Kh[illegible] | Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Science Meeting
Potential 3K clones on Master Plates.
~~B9320 grown on Hep 2.~~

From Page No.

am Science Review

Took transformants from yesterdays experiment from the incubator and put at 4°C.

Infected Hep.2 cells with "B9320 V_5 10^{-1}" 0.25ml/25cm² Flask.

Also infected Vero with the same stock - until/dil.

Meanwhile streaked out Potential 3K Clones on Amp⁺ Master plates.

Selected 10 clones each from Ncapsid/Vinun T/A Exp
5 clones each " " " Neil's large
5 " " " " " BRL large

Tues at Job Fair.

To Page No.

Witnessed & Understood by me, Syze W. Klub | Date | Invented by | Date

Recorded by David K Clarke

TITLE: Passage of CAT/RSV supernatant from transfection

Project No.____
Book No.____

From Page No.__

checked c.p.e of B9320 on Hep-2s and Vero.
Vero c.p.e. is extensive on undil infection

11-22-93 (P_3V)

pm. Infected 293 cells with "working stock" to check if they produce more virus – also passaged supernatant from transfected /CAT/RSV rescue Exp (Added ½ml of transfection super to 0.25ml of helper – high titer stuff) ~~V_3H_2 32°c.~~ or V_3H_1 32°c

Set up O/N mini preps on 8 potential 3Kb clones. – 100μg ml⁻¹ Amp.

Selected clones	Virion (V)	T/A 1-5	NC	T/A 1-5
	" "	Neils 13+14	"	Neils 13+14
	Virion "	BRL 19+20	"	BRL 19+20.

Harvested. Virus infected Hep2s – (B9320 V_5 H_1) undil

Veros – B9320 V_6 Dil

" " " undil. (extensive c.p.e.)

Phone calls out to potential R/As!

To Page No.____

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

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Project No.____
Book No.____
TITLE Harvest of CAT/RSV DI from Passage of Supernatant from transfection Exp. ~~Mini preps of potential 3K Clones.~~

From Page No.__

Carried out mini preps on the 18 potential 3K clones — went through procedure and stored DNA after EtOH pptⁿ — dried pellet and stored at −20°C — will Sca I digest etc tomorrow — will use ½ of total.

Also harvested 293 cell extract which had been produced from infection with rescued RSV/CAT/Helper (≈ 20h p.i.) — Froze away at −80°C. Also kept 1ml of supernatant for further passage.

3pm left for Dentist to have a tooth taken out!!

I don't think I'll be back today.

Will Bam HI/~~X~~ XhoI cut mini-preps — save 10µl of 50 for gel then Sca I cut ≡ 1400, ~900, ~700 } frags

To Page No.____

Witnessed & Understood by me, Serge W. Kble | Date | Invented by | Date

Recorded by David K Clarke

TITLE: Mini-prep analysis for 3Kb insert.
~~B9320 TCID~~$_{50}$

Project No.______
Book No.______

From Page No.___

Checked infected 293 cells – they are showing v. nice signs of c.p.e. – fusion, giant cells etc – will harvest later today

Will set up ~~BamHI~~ XhoI digest first (lower salt RXN) on ½ of total mini prep DNA prepared yesterday

Did BamHI cuts in 50µl with 1µl (20u) of Enzyme.

Gave 1.5hrs at 37°C – then φ chloroform extracted and ppt^{ed} at –80°C prior to XhoI digest^{n} – saved a little after double digest for analysis.

Carried out TCID$_{50}$ titrations on B9320 V_5 H$_1$ (undil infect^{n}); also B9320 V_6 10^{-1} and V_6 undil.

ScaI also cuts T/A vector to give (from M.C.S) fragments ≈ 2113 and 1819.

MON
642-5240
$175
10-30 – 3-30

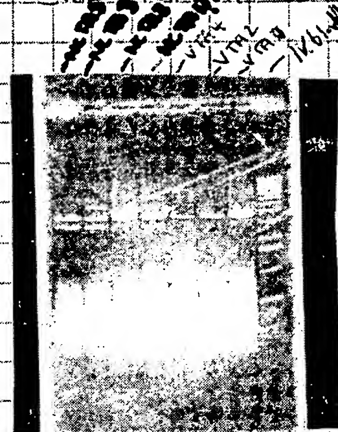
Set up RT/PCR RXN on NCapsid RNA (~1ml spun down) – resuspended in 3µl H_2O 3µl Bam > PM primer (160ngµl^{-1}) – heated at 92°C for 2mins → Ice (½µl Meth Merc – 5mins RT – ½µl 2-mercapto – 5mins R.T) Used 4µl of this mixture in Standard PerkinElmer Rev. Tran^{n} RXN followed by PCR. Extended program 44 72°C period to 7mins 30 cycles.

To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
Sy. W. Keble		Recorded by David K Clarke	

From Page No. 171

Meanwhile did BamHI digests of mini prep DNA – then, after ϕ etc. did XhoI digest – then analysed 10µl on a 1% Agarose gel. Stored rest at -20°C



Meanwhile harvested virus from P_3V infected 293 cells which were showing a lot of c.p.e. – froze down. Will do $TCID_{50}$ on Mon.

Also did $TCID_{50}$s on ~~P_3V H~~, 89320 V_5 H, undil
and 89320 V_6 10^{-1}/undil

\# 10^{-1} stock looked 'strange' – some EtOH may have leaked in and inactivated the virus!

NB Sca I digest of PCR II vector gives 2113 + 1819 bp frags if pre cut with EcoRI

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K Clarke

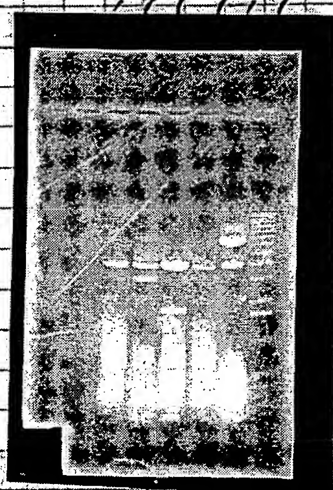
TITLE Further Mini-prep analysis for 3Kb insert..
Analysis of Long PCR RXN.

Project No.
Book No.

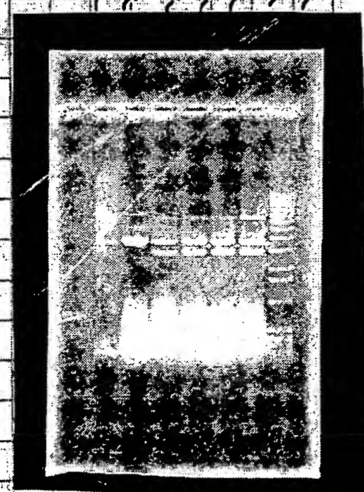
From Page No.

Prepared 2 x 1% Agarose gels for analysis of remaining BamHI/XhoI cut mini prep DNAs. Also loaded 10µl (of 100) from O/N RT/PCR RXN, to check for potential 3.8Kb product from nucleocapsid prep?

V-BRL-19, V-NE14, V-NE13, V-TA5, V-TA37, 1KB lad.



3.8Kb PCR, NC BRL 20, NC BRL 19, NC NE 14, NC NE 13, NC TA, 1KB lad



Results look V. encouraging - looks as though I have many correct 3Kb clones - will complete ScaI digestion analysis on Mon. Also 3.8Kb PCR product looks pretty good and should yield clones.

Cloroform extracted 3.8Kb RT/PCR Mixture and EtOH ppt^ed O/N at -20°. Will gel purify tomorrow.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature] David K Clarke	

Project No.

Book No.

TITLE Gel purification of 3.8Kb fragment

From Page No.

prepared 1% Agarose gel for Electro elution of 3.8Kb fragment.

Spun down RT/PCR mixture from yesterday - resuspended pellet in 15μl x1 Electrophoresis buffer and loaded on gel with marker ladder.

Designed primers for the 4K RT/PCR product

XhoI → BamHI 18mer mp = 54°C

cDNA primer. 5' TAC CAA GAG CTC GAG TCA

Bam → XhoI. 19mer mp = 54°C

5' AAT GGG ATC CAT TTT GTC C

The appropriate 3.8Kb DNA band (≈ 50-100ng) was eluted onto NA45 paper, rinsed briefly with NET buffer and collected in High salt NET (100μl - 2x 50μl rinses) - This was diluted x2 and mixed with 7μl of glycogen, then EtOH ppted O/N at -20°C.

To Page No.

Witnessed & Understood by me, Serge W. Kl[illegible]

Date

Invented by

Date

Recorded by David K Clarke.

Infection of TC 225s for N. capsid prep.

TITLE: Sca I digestⁿ of potential 3K clones

Project No. ____

Book No. ____

From Page No. ___

Ordered Oligos (see yesterday) from Ed - "No waiting" so should have them by Wed/Thur.

Checked $TCID_{50}s$ — Used wrong $TCID_{50}$ plates (cells were not confluent) - Other plate is now overgrown - will repeat the entire series on Wed.

Infected 10 x TC 225 (11-45am) - each with 1.5m of "V_3H_1 32°C" adsorbed 3hrs at 37°C then switched to 32°C

Set up Sca I digests of BamHI/XhoI digested mini prep.

Digested only those which appeared to have correct insert. Would predict the following bands

2,113 819	}	from PCR II Vector which has 1 Sca site
~1,000 ~900 ~700	}	from 3Kb insert

2-3hrs at 37°C. 30µl final vol 1µl (10u) enzyme

Carried out CAT assay on 20µl of cell extract from the passage of yield from the 1st transfection experiment. - let it run for ≈ 2½ hrs at 37°C. - Then froze down until tomorrow.

To Page No. ____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

Project No.____
Book No.____
TITLE Exp. design for differentiating between mixed Sub-type A and B RSV populations

From Page No.__

~10^6 89320 + 10, 100, 1000 'A' virus

Mixed with conc monoclonal antibody(s) and incubated at 37°C for 1-2hrs.

Need to establish plaquing efficiency of RSV 1st

Plate out total on $150cm^2$ flask under 1 to 3 diluted neutralising monoclonal(s).

Pick large selection of plaques

24 well plates in duplicate (Vero)

24 h.p.i. add differentiating monoclonals - then add Rabbit-anti mouse conjugated with enzyme

To Page No.____

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

From Page No.

Prepared 2 x 1% agarose gels - for analysis of Sca I digests

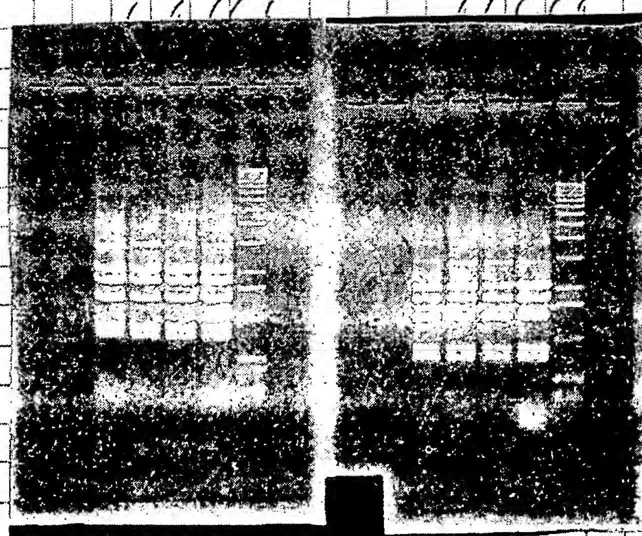
Checked infected TC 225's (Heps) - they are not showing v. significant c.p.e yet - will check again this afternoon ≈ 5pm

Loaded and ran agarose gels.

Carried out ethyl acetate extraction of CAT assay products. Dried down in a speed-vac - Meanwhile equilibrated the TLC tank with chloroform (190ml) : Methanol (10ml)

✓ NE14. / NC BRL R / NC NE14 / NC NE13 / 1Kb ladder.

NC TA5 / NC TA4 / NC TA3 / NC TA1? / 1KB ladder



V. good - all the clones look as though they contain the correct 3Kb insert.

It appears that the passage of the CAT activity did not work well (or at all) from the geiger counter - exposed o/N. Maybe fresh supernatant must be used. Will also try transfection with less RNA / more RNA

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Witnessed & Understood by me,
Date
Invented by
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Recorded by

Project No. -

Book No.

TITLE

From Page No. 177.

Set up O/N ligations at 12°C.

CON.	3.8Kb
2µl x 10 buffer	2µl x 10 buff
2µl (50ng) Vector.	[illegible]µl of DNA insert (~50ng).
15µl H_2O	5µl H_2O
1µl T_4 ligase.	1µl T_4 ligase

poured L-Agar-Amp$^+$ plates ($100\mu g\,ml^{-1}$) for tomorrow - stored O/N at 4°C.

Diluted XhoI → Bam. and Bam → XhoI (to $100ng\,\mu l^{-1}$) and stored diluted forms at -20°C stocks at -80°C.

To Page No.

Witnessed & Understood by me, Serge W. [illegible]

Date

Invented by

Date

Recorded by David K. Clarke.

Nucleocapsid Preps.

TITLE: Bacterial transformation with 3.8Kb fragment.

Project No.

Book No.

From Page No.

see reference binder #22A

~~Re~~developed CAT Assay ant[illegible] to see if any CAT activity had been passaged — No luck — will repeat with fresh supernatant next time (probably next week) — pre-infect with helper?

Ran through N.capsid preparation protocol on 10 x TC 225s — which were showing good c.p.e (fusion etc) — Lysed in a total vol. of 60ml — spun out nuclei — resuspended in 9.5ml total vol of 15% K^+ tartrate — loaded onto preformed 55%-45%-35%-25% gradients in SW 50.1 tubes. Ran at 29K, 5°c for 16hrs (on at 6-30pm Wed) — off at 10-30am.

Meanwhile carried out $TCID_{50}$ on V_5 H, B9320 undil.
V_6 B9320 dil/undil.
and V_3 293,(A2)

Also did bacterial transformation — T/A cloning kit cells using 5µl of Control/and 3.8Kb ligation mixtures for each Transformation. Plated out on L-Agar (100µg ml⁻¹ Amp)

NB Failure to Phenol extract eluted DNA may prevent efficient ligation!!!

To Page No.

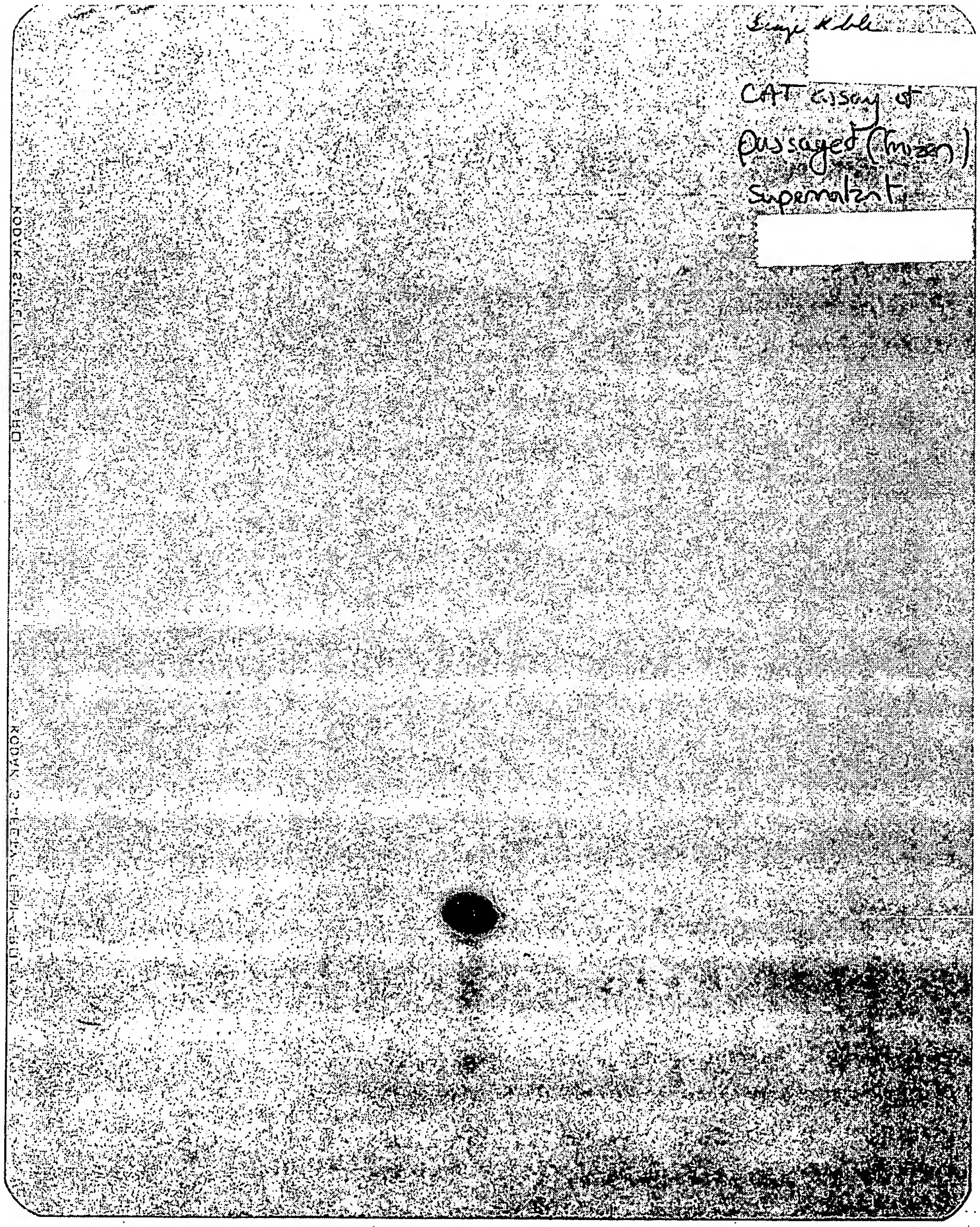
Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by David K Clarke


CAT assay of
passaged (frozen)
supernatant
KODAK

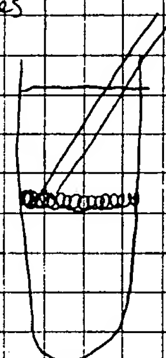
Project No.______
Book No.______

TITLE Harvesting N.capsid prep. O/N minis of potential 3.8Kb clones

From Page No.__

Checked Potential 3.8Kb clones – it looks as though there may be a higher than background level of white colonies – indicating the presence of insert DNA!

Also took off N.capsid spin – there were dense flocculent bands ½ way down the centrifuge tubes



Harvested – pelleted in Eppendorf tubes – resuspended in NTE – added SDS (0.2% final conc – and Proteinase K – 1mg ml^{-1} final conc.

Let digest at 37°c for 1-2hrs
Then phenol cloroform extracted – (x2 if necessary)

ETOH ppt^{ed} – Noted contamination with chromosomal DNA

Will run an agarose gel tomorrow to take a look at it

Set up 12 Mini preps (mostly v. small white colonies were selected) 8µl of 50µg ml^{-1} Amp / 4ml L-broth – Also streaked onto a Amp$^+$ Master plate for O/N growth at 37°c.

Can check potential 3.8Kb clones by BamHI / XhoI ~~[illegible]~~ – neither site should exist in the 3.8Kb clone

To Page No.____

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K. Clarke

Checked N capsid Prep-on a Gel.

TITLE: RT/PCR on 4Kb fragment/DMSO/Formamide etc.

Project No.

Book No.

From Page No.

Checked growth of Mini preps - Many had not grown - will re-do sum → Mon. from well grown master plates.

Spun down ½ml of New N capsid prep - resuspended largish pellet in 10µl x 1 MOPS gel buffer - 10µl of Deionised Formamide - added µl of 1mg ml⁻¹ EtBr and heated at 65°C for 5mins - proceeded to load gel, including some RNA marker.

Checked RNA gel - there is no clear indication that there is any genomic RNA there - will run a sample through RT-PCR to check if any product is produced.

Set up 4 different RT/PCR RXNS. - spun down ~1.3ml x 2 for 'Old' genomic RNA prep - 1ml for New Prep - resuspended each pellet in 3µl H_2O + 3µl primer - 92°C - 2mins - ½µl Meth Merc - 5mins - ½µl Mercaptoethanol - 5mins → TOOK 4µl and set up a

1. Reg RT/PCR with 'Old' prep
2. RT/PCR + 5% DMSO 'Old' prep
3. RT/PCR + 3% Formamide/5% Glycerol 'Old prep'
4. Reg RT/PCR with New N capsid prep

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kble		Recorded by David K Clarke	

Project No.

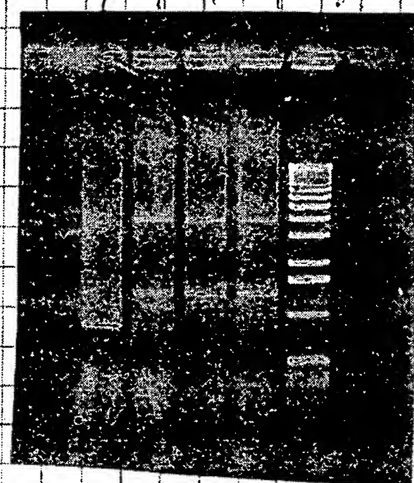
Book No.

TITLE Gel analysis of 4kb RT/PCR products

From Page No.

Prepared a 1% agarose gel for analysis of potential 4.0kb RT/PCR products (Elongation time - now up to 8mins)

Loaded 1/10th of each reaction + DNA marker ladder



Will gel purify the 4kb fragments tomorrow

Meanwhile - cloroform extracted and EtOH ppt^ed

Good - it looks as though the 4kb RT/PCR product has been produced ≈ 5-10ng (μl⁻¹). The New Ncapsid prep is not working though. The presence of DMSO or formamide/glycerol did not appreciably affect yield.

Total yield will be ≈ 150-300ng in 3 reactions.

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Recorded by David K Clarke

TITLE: Gel purification of potential 4K DNA fragment
Set up O/N minis on potential 3.8Kb

Project No.

Book No.

From Page No.

Set up O/N mini preps on a selection of potential 3.8Kb clones
(4ml - 100μg/ml Amp ≡ 8μl of stock)
Will do DNA extractions tomorrow.

Also spun down 4K RT PCR products - redissolved in 15μl of x1 TAE Elect. Buffer
and loaded onto a 1% Agarose gel, alongside marker I.

Proceeded with electro elution - Phenol chloroform was extracted after
elution and ppted with 8μl of glycogen Co-ppt. - There could be
anywhere from 25-100ng final yield of 4Kb fragment. Will ligate
total with 2μl (50ng) of vector

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kobe		Recorded by David K Clark	

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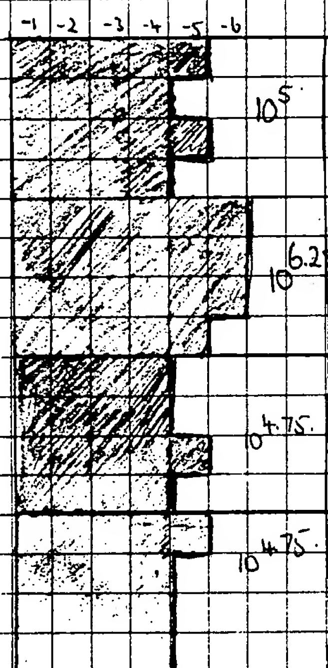
Project No.______ Book No.______

TITLE Mini-preps of 5 potential 3.8Kb clones
Restriction analysis of these 5.

From Page No.___

Checked growth of mini preps – only 5 look as though they have grown – will extract DNA later today

Did final read on $TCID_{50}$



10^5 B9320 V_5H_1 Undil.

$10^{6.25}$ B9320 V_6 Undil

$10^{4.75}$ B9320 V_6 Dil.

$10^{4.75}$ (A2) V_3 293,

Started mini preps – took through to Dried DNA pellet stage – then set up Bam H I digest in 50µl – $1\frac{1}{2}$µl enz + $\frac{1}{2}$µl RNase A.

1-2hrs at 37°c. Then proceeded to analyse on a 1% agarose gel

To Page No.______

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K Clarke

000208

From Page No. 184.

Good - looks as though N° 4 may contain the 3.8Kb insert (vs the 3.93Kb) Vector - Proceeded with Cla I digestion of the BamHI cut mini prep DNA. - Should look for a 659 bp and a 3141 b.p fragment, with the vector remaining unchanged.

Set up O/N ligation of gel purified 4Kb material with 4Kb PCR II Vector.

+ Control

2µl x10 buff.
2µl Vector (50ng)
15µl H_2O or DNA 4Kb insert.
1µl T_4 DNA ligase
20µl — O/N at 12°C.

To Page No.____

Witnessed & Understood by me. [signature] Date

Invented by ______ Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Cla I cut analysis of potential 3.8Kb clone.
Transformation with potential 4Kb ligation products

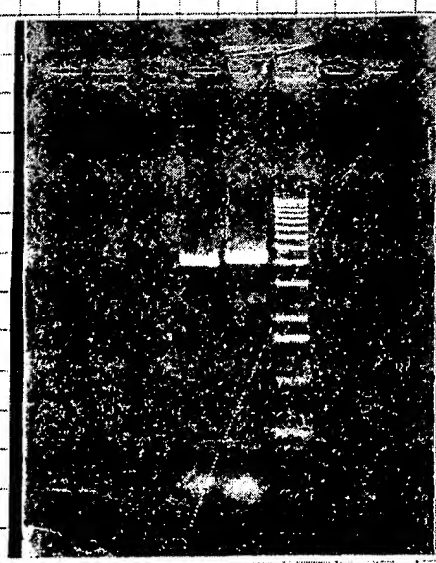
From Page No.

Spin down Bam HI cut Mini prep DNA - and set up Cla I digestion in 40μl — 1-2hrs at 37°C. - prepared mini-gel (1%) for analysis.

Checked P_3 Vero stocks - have sufficient for another N-capsid prep - will order Hep 2 cells for Mon.

Ordered 1Kb DNA markers from Gibco/BRL

Started transformation with T/A cells - Mixed 5μl of each ~~trans~~ ligation Mixture with the competent cells and left on ice for ≈ 1hr before heat shocking at 42°C



Cla I digest didn't appear to work, indicating that this the wrong clone, or that the Cla I site may have been lost due to random mutation.

To double check this clone set up 1hr HinD III digestions (which has 3 cut sites in the 3.8Kb fragment.

HinD III digests did not indicate any insert either

To Page No.

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[signature]		Recorded by [signature: M. David K. Clarke]	

000210

From Page No. 186

Electroporated in 1µl of DNA from Con/4K6 ligation mixtures

Used 25µl of electrocompetent cells/transformation – used 1.8KV at 25µF capacitance (200 Ω resistance)

Added ≈ 1ml of SOC to each cuvette imm. after electroporation and resuspended Bact cells in this – then incubated at 37°C for ≈ 1hr – plated out 200µl on Amp+ IPTG+ X-Gal+ plates

To Page No.

Witnessed & Understood by me, Aaje W. Khale | Date | Invented by | Date

Recorded by David K Clarke

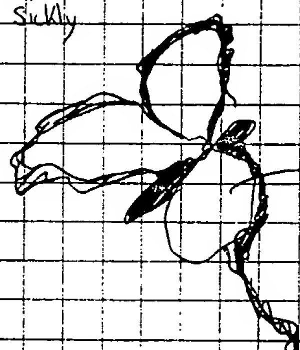
Project No.
Book No. TITLE

From Page No.

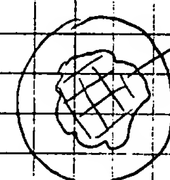
Checked bacterial transformants — looks pretty good — the electroporation seems to have worked pretty well — there are quite a few colonies on the 'test' plates but not on the CON — ditto T/A.

Put at 4°C to let Blue colour develop, before streaking onto Master plates.

Meanwhile - infected 293 cells with RSV $V_3 H_2$ 32°C ($10^7 ml^{-1}$) — the cells were V. delicate and looked as though they would come off the plate! — Then proceeded with transfection — under the microscope the cells looked very sickly



lacy pattern - cells all contracted after addition of virus. cells around the edges had come off



remaining cells (v. sick looking)

Meanwhile streaked out 36 potential 4K6 clones from electroporation, and 36 potential 4K6 clones from T/A cloning — + 2 control (-ve) Blues

Removed Optimem / transfectace / RNA mixture and added 1ml of MEM and left O/N

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Sage W. Kobble

Recorded by David R. Clarke.

Project No.
TITLE: Book No.

From Page No.

Checked restreaked master plate for potential 4K clones - a few clones are not growing well - however there will be plenty to screen - will drop Amp conc to 50μg ml⁻¹ in O/N cultures.

Meanwhile prepared extracts from transfected 293 cells - as per protocol except prepared final extract in 50μl Tris (not 100μl) since the number of cells was dramatically reduced.

Also did a freeze thaw on one set of transfected cells, to see if that permitted improved transfer of CAT activity to fresh 293 cells (which were pre-infected - 1hr - with 0.5ml of V_3H_1 32°C ≈ 10^7 pfu ml⁻¹). The preinfecting inoculum was removed prior to addition of transfection supernatant.

Set up 22 mini prep cultures (2 -ve controls); 10 from T/A cloning Exp, 10 from Electroporation experiment

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Koble		Recorded by David K Clarke	

Project No.
Book No.
TITLE Mini prep analysis of potential 4Kb clones.
RT/PCR of potential 3.8K fragment

From Page No.

All O/N mini-preps grew – so started mini preps and took through to EtOH ppt^n stage; then prepared a 1% agarose gel to do quick CCC analysis along with -ve con and 3Kb insert con.

Looked at a total of 18 minis – none contained the 4K insert.

Will screen more minis on Mon – if that doesn't work will do XhoI/Bam digest^n on PCR product prior to gel purification. Will also do Bam digestion on 3.8Kb fragment prior to gel purification. (also on vector appropriately).

will not do phenol chloroform initially.

Meanwhile prepared cytoplasmic extract from 293 cells which had been infected with 1st passage supers (freeze thawed super or No freeze-thaw super). Final cell extract ~~contained~~ was 100µl. — rapid freeze → -80°C.

Also set up reverse transcription on 1ml (spun down) of Nucleocapsid prep. – resuspended in 2µl H_2O/2µl primer – heated at ≈ 80°C 2' → ice, then mistakenly added 1µl 2-Mercaptoethanol (mistakenly!)! – proceeded with rev. transcription anyway ≈ 1.5hrs at 42°C

heat inactivated at 90-95°C for ≈ 2mins

Then proceeded with PCR rxn.

NB Can use KpnI to linearise Vector + Insert.

To Page No.

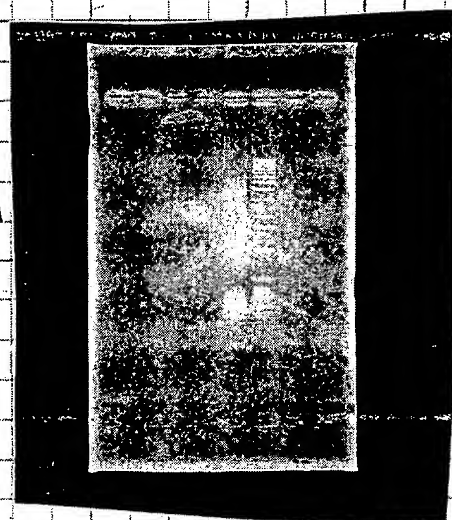
Witnessed & Understood by me, [signature]
Date
Invented by
Date
Recorded by David K Clarke

Project No.____
TITLE Gel Analysis of 3.8Kb RT/PCR RxN. Book No.____

From Page No.__

Prepared 1% Agarose gel for analysis of RT/PCR products (Hopefully 3.8Kb fragment) Loaded 10µl (of 100µl) for analysis

Will have not more than 25ng of 3.8Kb band after gel purification - will ligate with 25ng of Vector in 10µl



Surprisingly there is a 3.8Kb PCR product! Phenol chloroform extracted and EtOH ppt'ed with 3µl of glycogen as Co-pptnt. Will try to gel purify tomorrow after Bam HI digestion (will also Bam digest same Vector)

The Bam HI digestion should improve the cloning efficiency of the 3.8Kb frag

To Page No.____

Witnessed & Understood by me, Sage W. Kohle | Date | Invented by | Date

Recorded by David K. Clarke

Project No.

Book No.

TITLE BamHI digestion and gel purification of 3.8Kb PCR prod. and PCR II cloning Vector

From Page No.

Spun down 3.8Kb RT/PCR product - dried - and set up BamHI digestion in 20µl ; along with PCR II Vector - gave 1.5hrs at 37°C (used 1µl Bam for each digestion)

Meanwhile prepared a 1% agarose gel for electroelution - loaded and ran for ≈ 45mins with marker DNA ; - Proceeded to electroelute DNA onto NA-45 paper - 6mins - a little too short for the PCR II Vector - only got ≈ ½ (100ng) - Eluted into 120µl total of high Salt NET - Diluted x2 and Phenol Cloroform extracted - then ppt^ed with 10µl of glycogen as Co-ppt^nt. Would estimate max recovery at 25-50ng for 3.8Kb band and 100ng for Vector - will set up 1:1 ratio for ligation. (all of 3.8Kb material ≈ 25ng with ¼ of Vector in 15µl ligat^n Rxn).

Meanwhile set up another 24 minipreps, with Potential 4K clones - Amp^r - Will run minis tomorrow

Need a new book!

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

TITLE

Mini prep analysis of Potential 4Kb. clones.
CAT assays of P_0 and P_1 RSV/CAT transfections - A helper.

Carried out another 24 mini preps on potential 4K clones — prepared 2x 1% Agarose gels, each with 2 combs. Omitted phenol chloroform extraction to save time. Ran 1/5 of each prep - no luck - there were no 4K-insert-sized plasmids.

Meantime infected Veros (new) with 'VP6 B9320 und.1' - did an undil + 10^{-1} dil infection (0.25ml on 25cm² Flasks) - adsorbed ≈ 3hrs - then fed with 4ml of MEM.

Also carried out CAT assays using the four previously prepared cell extracts
1. 1/2 RNA (reduced amount of transfecting RNA.
2. No freeze Thaw.
3. No freeze ~~thaw~~ passage 1.
4. Freeze - thaw passage 1.

Used 20µl of Extract for each reaction - let run for 2-3hrs at 37°C - froze away at -80°C prior to ethyl acetate extraction and TLC.

Witnessed by: Serge W. Kibbe

Recorded by David K Clark

DATE

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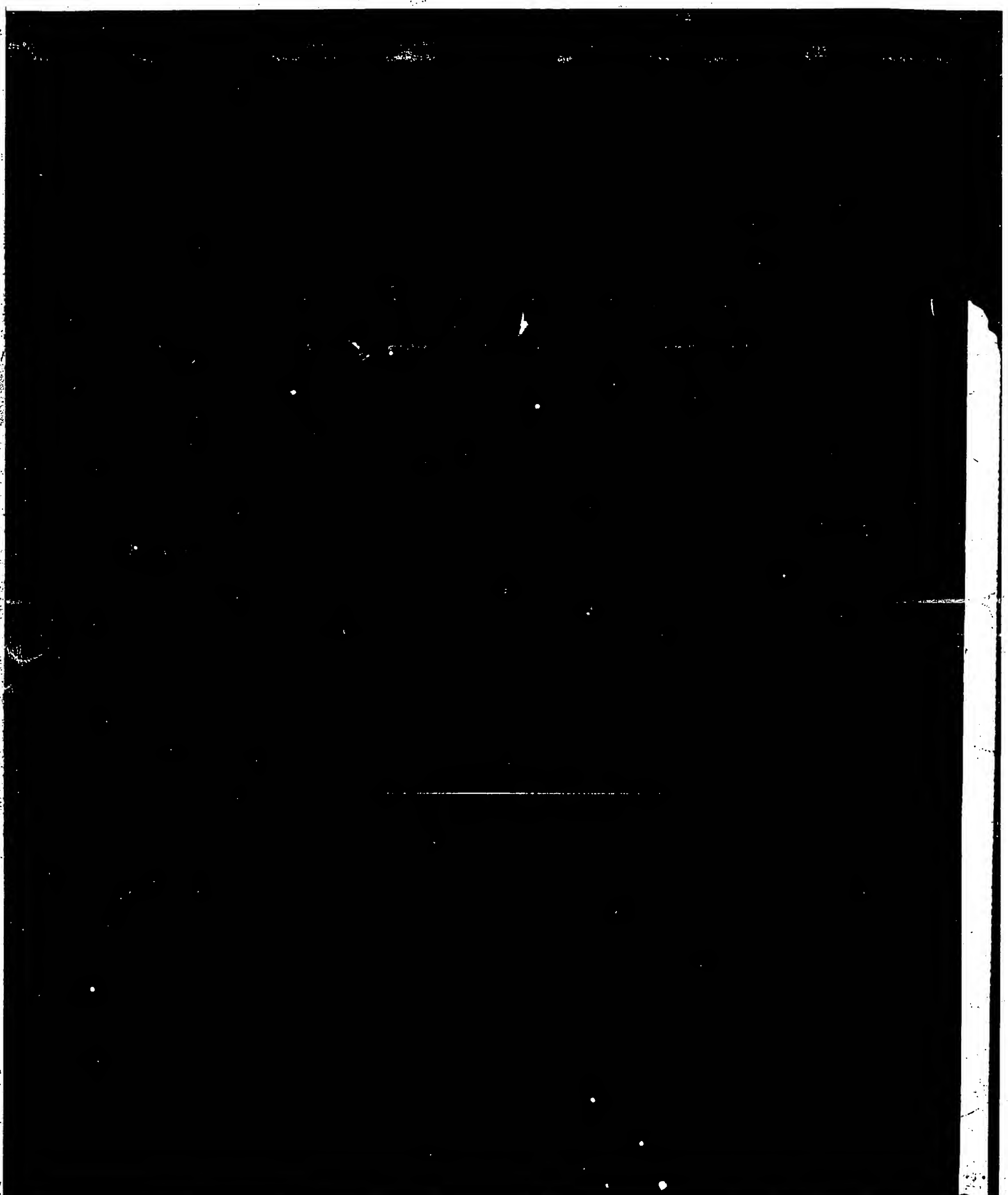

Book 2

DAVID CLARKE

Associated Binder #045A

NOTEBOOK NO. #045

ISSUED TO DAVID CLARKE

ON

DEPARTMENT

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INSTRUCTIONS

1. **The primary purpose of this notebook is to protect your and the Company's Patent-Rights by keeping records of all original work in a form acceptable as evidence if any legal conflict arises.**

2. • When starting a page, enter the title, project number, and book number.
   • Use <u>ink</u> for permanence -- avoid pencil.
   • Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
   • Avoid making notes on loose paper to be recopied.
   • Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.

3. • Give a complete account of your experiments and the results, both positive and negative, including your observations.
   • Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
   • Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.

4. • After entering your data, sign and date the entries.
   • Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
   • Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.

5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.

6. This notebook and its contents are the exclusive property of the Company. It is confidential and the contents are not to be disclosed to anyone unless authorized by the Company. You must return it when completed, upon request, or upon termination of employment. It should be kept in a protected place.
   If loss occurs, notify your supervisor immediately, and make a written report describing the circumstances of the loss.

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TITLE: TLC analysis of CAT Transfections.

Project No.______
Book No.______

From Page No.___

Ethyl Acetate extracted 4 CAT assays from yesterday; dried down in the speed Vac — meanwhile equilibrated TLC Tank with solvent.

Checked c.p.e. on 293 cells — nothing much yet — will check again this afternoon

Loaded TLC

LHS → RHS

$\frac{1}{2}$ RNA   No F/T   F/T 1st Passage   F/T 1st Passage

Took off after ≈ 2 hrs — and put away for autoradiogram.

From the sound of the Geiger counter, there should be significant CAT activity.

Meanwhile prepared X6 Amp^+ agar plates $100\mu g\,ml^{-1}$
X3 Kan^+ agar plates $50\mu g\,ml^{-1}$
poured - dried and stored at 4°C until tomorrow.

Meanwhile set up O/N ligations with Bam HI cut 3.8K RT PCR product which had been gel purified. Used all in single ligation

C/V	3.8K	O/N 12°C
2μl Vector (25ng)	2μl Vector	
1μl x10 Lig	1μl x10	
6μl H_2O	6μl insert	
1μl T4 ligase	1μl T4 ligase	
10μl	20μl	

To Page No.___

Witnessed & Understood by me, [signature]   Date

Invented by   Date

Recorded by David K Clarke

Project No.______
Book No.______ TITLE Transformations with 3.8K RT/PCR product

From Page No.__

Checked c.p.e on 89320 infected Vero cells - progressing nicely - will harvest later today

Developed RSV-CAT Transfection autorad - looks good

① There was a v. high CAT activity following the initial transfection - even when the transfecting RNA was cut in half.

② CAT activity can be passaged either directly from supernatant from transfected cells - without any processing, OR following freeze-thaw of transfected cells

Set up transformations - 5µl of each ligation (con + 3.8K) was used to transform T/A cells

2µl of each ligation was used to transform Electro cells

Meanwhile harvested 89320 D.I./Undil. C.p.e. was extensive in both, but was a little more pronounced in Undil.

Froze down at -80°C.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Sgr W. Kolk		Recorded by David K Clarke	

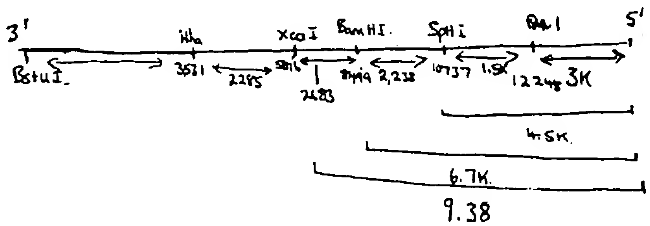
TITLE: Planning future work (for Cloning Viral stocks etc.)

Project No.

Book No.

From Page No.

Checked for bacterial transformants — No Joy - only one transformant was present on an A/T plate — will check it out by mini prep tomorrow.



Revised cloning procedure for genome of RSV.

prepared Amp^r plates to restreak clone banks.

Checked RSV A2 ATCC P_1V, P_2V etc. — will order.

1 x 75cm² Vero for $P_1 \rightarrow P_2$ Vero (ATCC)
2 x 75cm² Vero for $P_2 \rightarrow P_3$ Vero (ATCC)
2 x 25cm² Vero DC5 for $P_1 \rightarrow P_2$ Vero / $P_2 \rightarrow P_3$.

Designed SphI → PstI cDNA primer

5'-ACA/TAT/AGG/CAT/GCA/CC~~X~~-3'   17/~~18~~-Mer
50°C m.p.

Plated out clone banks and grow O/N at 37°C.

Also set up single mini prep of potential 3.8K clone.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature]	

Project No.____
Book No.____

TITLE Mini prep analysis of potential 3.8K clone
TCID50 for 89320; Attempted Plaque Assay

From Page No.__

Carried out mini prep DNA extraction on potential 3.8K clone - ran a sample on a 1% Agarose gel alongside a 3K control. No-luck - it did not contain a 3.8K insert - will resort to cloning smaller fragments.

Meanwhile did $TCID_{50s}$ on 89320 - V_7 Dil/Undil

Also infected $25cm^2$ Vero monolayers with 10^{-2}, 10^{-3} and 10^{-4} Diluted P_3V ATCC for plaque assay (used 200µl of each) - Adsorbed 2-3hrs and then overlaid each with 6-7ml of 0.4% Agar/EMEM. Allowed to solidify at Room Temp for 20mins, then placed at 37°C.

Ordered Sph I → PmL I Oligos

Franks Meeting at 4pm.

To Page No.____

Witnessed & Understood by me, Serge W. Kbh... | Date | Invented by | Date

Recorded by David K Clarke

Project No.____

TITLE:________________ Book No.____

From Page No.__

Prepared Alex's letter of recommendation for Graduate schools

Stored bacterial clone banks at 4°C.

Checked Pt. balance in viv. plaque assays. — seems fine.

To Page No.____

Witnessed & Understood by me, Serge W. Koble

Date

Invented by

Date

Recorded by David K Clarke

Project No.______

Book No.______ TITLE Infection of Hep-2s for Nucleocapsid prep.

From Page No.__

Ordered 3 x 96 well plates for Fri. to titrate Vero stocks.

Infected 10 x TC150s with 1ml each of either P_3V or "P_3H, 32°C"

adsorbed ≈ 3hs at 37°C 10am - 1pm. then put at 32°C for 48hs

prepared NA 45 paper for electroelution of DNA bands - stored at 4°C

Spent the afternoon preparing 2-Monthly report.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Sze W. Kolb		Recorded by David K Clarke	

Project No.

TITLE: General virology observations. Book No. 7

From Page No.

Checked $TCID_{50}$ for V_7 B9320 Undil/Dil (4 days p.i.)

It is clear that Undil is still better than dil for infection. It looks as though the titer is going to be $\approx 10^{5.5}$ for Undil (lower for dil) Will make a final reading on Thurs. Should try infecting Veros at 32°C and/or infecting Vero DC 5 at 37°C/32°C all with P_6 or P_7 V B9320.

Checked plaque assay of A2 P_3V — small plaques are now visible with the naked eye — will leave until thurs to get a final count.

Checked c.p.e. progression on Hep-2 cells at 24h.p.i. — There are some signs of c.p.e. on the Hep 2s will check again this afternoon and hopefully they will be ready to harvest by tomorrow — late morning.

Prepared some new 55% w/w K⁺ Tartrate.

Will soon order

✓ SphI to BamHI 5' — GGT/GCA/TGC/CTA/TAT/GT 17mer 50°C m.p.

✓ XcaI to BamHI 5' — TGG/[redacted]/TTG/GTA/TAC/CAG/TGT/T 19mer 54°C m.p.

✓ HhaI to XcaI 5' — TTT/ACC/ATA/TGC/GCT/AAT/GT, 20mer 54° m.p.

✓ XcaI to HhaI 5' — AAC/ACT/GGT/ATA/CCA/ACC/A, 19mer 54°C m.p.

To Page No. 8

Witnessed & Understood by me,	Date	Invented by	Date
[illegible]		Recorded by David K. Clarke	

[redacted]000246

Project No.

Book No. TITLE

From Page No. 7

✓ BstUI to HhaI

GT↑

5'- ACG/CGA/AAA/AAT/GCG/TAC/A 19mer 54°C m.p

✓ HhaI to BstUI

Should put in a restr. recognit site.

5'-ACA/TTA/GCG/CAT/ATG/GTA/AA 20mer 54°C m.p

Prepared K^+ tartrate solution + lysis buffer for Nucleocapsid prep.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Angus W. Kolah		Recorded by David K Clarke	

TITLE: Nucleocapsid Preparation

Project No.
Book No.

From Page No.

Proceeded with nucleocapsid prep - (did Lysis step in 10ml/5 TC150s)

Infected 3x 75cm² Veros → not very confluent!

Two with P_2V ATCC } 0.25ml inoculum each.
One with P_1V ATCC }

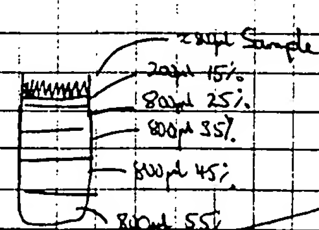
Also infected 2 x Vero - DC-5

~~both~~ with V_6 B4320 Undil → 37°C
→ 32°C.

Screwed up N.capsid prep - forgot to spin out nuclei.

However - tried to compensate by addition of RNase free DNASE (to digest nuclear DNA. (mixed in 10µl per tube prior to T.E spin) - Resuspended each pellet in 2mL 10mm Tris, 10mM $MgCl_2$ - added another 5µl of DNase and incubated on ice for 2-3 hrs - after 1hr added 500µl of 55% K tartrate.

Meanwhile prepared gradients.



To Page No.

Witnessed & Understood by me, Date Invented by Date

Sigr W. Kble

Recorded by David K. Clarke

Project No.______
Book No.______ TITLE N capsid spin off, RT.PCR 1.6K

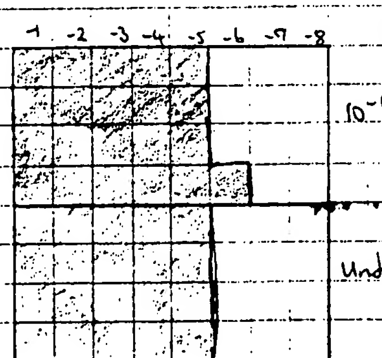
From Page No.___

Checked plaque assay 6 days post infection - plaques are well developed - stained with Crystal violet after flicking out the agar. The plaques are uneven and should probably have been stained at day 5 p.i. (possibly day 4) - also should perhaps use 6ml (instead of 5ml) per 10ml MEM to decrease sloppiness slightly. Titre was ≈ 3×10^6 pfu ml^{-1} calculated from 10^{-4} dilution. This value was slightly higher than that obtained for the $TCID_{50}$ (as expected).

Took off nucleocapsid spin - Presence of genomic ~~RNA~~ DNA does seem to be a problem - harvested the white, flocculent minor band close to the major band of material - spun down in eppys - SDS-Prot K treated pellet - phenol Chloroform extracted and EtOH ppt^ed.

Meanwhile spun down RNA (genome) preps for cDNA/PCR rxns.

Did final read on $TCID_{50}$s B9320 P_7V - it appears that infection with P_6 Vero 10^{-1} gave a slightly higher titre vs P_6 Vero Undil.



Also checked DC-5 infected Veros 32°C/37°C - both are showing c.p.e as are the Veros infected with ATCC P_1 and P_2 Vero.

To Page No.____

Witnessed & Understood by me, Serge W. Kibbe[?] | Date | Invented by | Date
Recorded by David K. Clarke

From Page No. 10

Set up RT/PCR runs on three different samples - using SphI > PstI as primer (m.p 50°C - 17mer) (a 1.5K frag)

Reduced 72°C phase to 5mins (for the shorter fragment).

Did another check on c.p.e.

V. noticeable that 37°C B9320 until P_6V infection has much more pronounced c.p.e. than 32°C infection.

To Page No.

Witnessed & Understood by me, Date Invented by Date

Serge W. Kibler

Recorded by David K Clarke

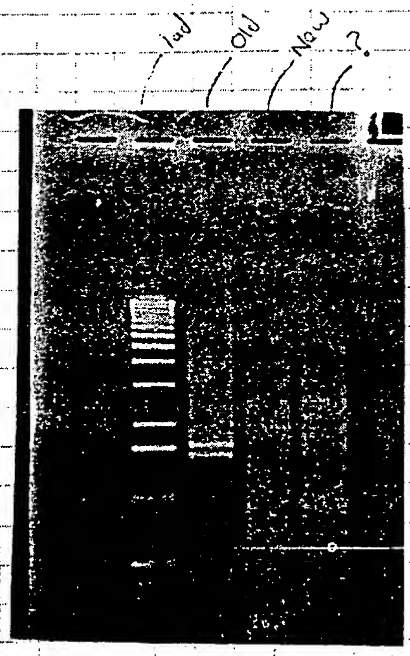
Project No.
Book No.
TITLE Analysis of 1.5K RT/PCR RxN

From Page No.

Checked c.p.e on infected Veros - all looks good - will harvest later this afternoon. c.p.e looks v. good on DC-5 at 37°C

Took off RT/PCR RxN. Loaded 10μl samples each of

(LHS) Lad/Old/NEW/?/



The lower PCR band is the one that I want - will probably purify both and select later, or can set up electroelution to enrich for lower band i.e. run out as far as possible, then elute for a short period of time (3-5 mins).

Cloroform extracted and EtOH ppt^ed with 5μl of stock glycogen as co ppt^nt - will gel purify on Sun.

Would estimate 200-500ng in 1.5K band. Should give 100-250ng following purification.

Proceeded with virus harvests and TCID 50s.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No:
TITLE: Book No.

From Page No.

Spun down RT/PCR for 1.5K fragment – Prepared 1% Agarose gel for purification of DNA.

Ran out – long run – stained – eluted DNA onto NA45 paper then eluted off in high salt – Phenol/chloroform extracted and ppt^ed with glycogen.

Decided to harvest both DNA bands onto NA45 paper (1 piece) – just in case! Also will add bulk – minimising losses.

Trimming out cloned DNA with EcoRI should give 3 fragments. 3.8K vector
600 b.p frag.
924 b.p. frag.

Ordered 4 Oligos indicated on page 7.

To Page No.

Witnessed & Understood by me, Serge W. Kohle | Date | Invented by | Date

Recorded by David K. Clarke

Project No.

Book No.

TITLE Infection of Heps for Nucleocapsid. O/N ligation of 1.5K Frag.

From Page No.

Ordered T/A cloning Kit.

Infected 10 x TC150s each with ≈1ml of either V_3 ≈ or $V_3 H_1$ ATCC

absorbed 3hrs at 37°C – then put at 32°C.

Meanwhile set up o/n ligation

Resuspended purified 1.5K material in 8µl H_2O

Set up RXNs.

2µl Vector (50ng) 1:1 ratio
2µl Insert (≈25ng)
~~2µl x10 buffer~~
13/15µl H_2O (Test/con)
1µl T_4 DNA ligase

20µl → O/N 12°C.

prepared 9 Amp^+ plates for transformation. – Poured – stored at 4°C.

Prepared X-gal (40mg ml⁻¹) in Dimethyl formamide – stored at -20°C in ~~sil~~ silver foil wrapping

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kibble		Recorded by David K Clarke	

000253

TITLE: Transformation of Bacteria with 1.5K fragment.

Project No.

Book No.

From Page No.

Checked c.p.e on infected Hep-2s – Those infected with V_3H_1 are showing a higher level of dead cells than those infected with P_3V where there is not much indication of viral c.p.e yet. Will check again later this afternoon.

Prepared fresh lysis buffer for nucleocapsid prep.

Preliminary check on $TCID_{50s}$ indicates

(1) DC 5 Veros are not significantly better for growing B9320.
(2) 32°C may be better than 37°C for growing B9320 (should be tried on 'regular' Veros

Titres

Will order some Hep-2 cells for V_3H_1 stocks. $3 \times 75cm^2$

Also some regular Veros ~~+~~ for B9320 32°C growth cf 37°C growth.
Dil/well at each temp. $4 \times 25cm^2$

Carried out transformation of 'One-shot' bacteria from Invitrogen.
Used 5µl from each O/N ligation (plastic residue from Dorothy formamide dissolution of plastic container used to make up X-gal may inhibit bacterial growth!)

To Page No.

Witnessed & Understood by me, Serge W. Kohler | Date | Invented by | Date

Recorded by David K Clarke

Project No.
Book No. TITLE Nucleocapsid Prep.

From Page No.

Virus infected Hep-2's were showing a lot of cpe - initiated Nucleocapsid production - including NaCl/Nucleus spin.

Checked bacterial transformants - there are some whites on the test plate - less on the control - will streak out on a master plate O/N.

Ran through nucleocapsid prep - resuspended each crude N. capsid pellet in 2.4ml 15% (w/w) K^+ tartrate - Meanwhile prepared K^+ tartrate gradients

Did lysis steps with ~~Sat/pellet~~ 10ml 5 TC150s.

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by

000255

TITLE: Nucleocapsid harvest / O/N Minis on Pst 1.5K clones

Project No.

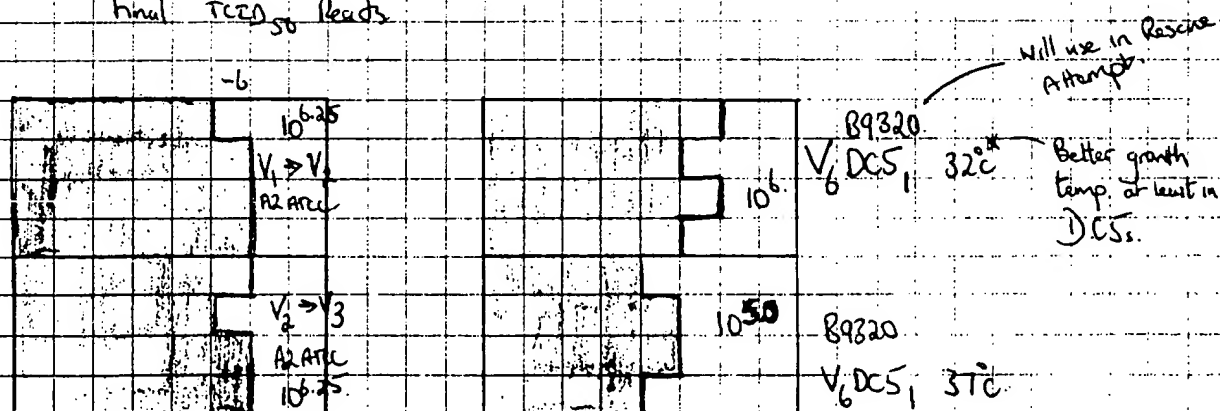
Book No.

From Page No.

Checked transformants (streaked on Master plate) About ½ are showing some signs of blue colour - indicating absence of an insert - will pick a few bluish ones + the remainder of white ones and set up O/N Mini preps - Did 14 minis (~4 or 5 look as if they may be +ves)!

Meanwhile took off nucleocapsid spin and harvested bands. Resuspended 5 flasks worth in 1ml final vol T.E. buffer and carried out SDS/proteinase K digestion for ~1hr at 37°C followed by phenol Chloroform extraction and ethanol pptⁿ.

Final $TCID_{50}$ Reads



These titers are good, and consistent with previous titers at the same passage.

Diluted out and stored new Oligos.

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David R. Clarke

Project No.

Book No.

TITLE Mini-prep analysis

From Page No.

Carried out mini prep analysis of 14 possible 1.5K clones – 7 and 21 had not grown v. well, so can expect to get DNA from only 12 (of those 4 had blue colour tint – so narrows field down to 8 possibles.

Meanwhile prepared a 1% gel for analysis of mini preps

Ran the gel while in meeting with Laura Coruzzi – unfortunately it ran too far – Proceeded to Phenol Chloroform extract the mini preps and then etoH ppted

Spun down new N capsid prep RNA – it looked good – dried and stored at -20°C in preparation for reverse transcription/PCR tomorrow.

meanwhile spun down plasmid DNA from mini-preps and set up EcoRI restriction digests in 10μl final vol. ~1μl RNase A and 1μl EcoRI ~1hr at 37°C.

Loaded half of each digest on a 1% Agarose gel with a 1Kb ladder – checked gels – No luck – there does not appear to be any inserts at all.

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by David K Clarke

Project No.

TITLE RT/PCR for BamHI/SphI fragment.

Book No.

From Page No.

Set up RT/PCR Reactions on RNA from newest nucleocapsid preps (V_3, and V_3H_1 derived nuc.)

Resuspended each pellet in 3µl H_2O and 3µl Bam → Pml primer – 90°c for 1.5 mins → Ice → ½µl of Meth.Hg → RT for 3mins → ½µl of β-mercap. → RT for 3mins → Rxn mixture for 1-2hrs with Rev. Tase, 42°C.

Heat inactivated at ≈95°C for 2.5 mins – then proceeded to set up PCR RXNs using ~ 0.5 - 1.0µl of Taq.

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by

000258

Project No.______
Book No.______
TITLE 1.5Kb ligation

From Page No.__

Set up O/N ligation of gel purified 1.5K frag and PCR II vector

2µl of Vector
4µl Insert (Saved 2µl for gel analysis)
2µl x 10 buffer
11µl H_2O
1µl T_4 DNA ligase

20µl. put at 12°C O/N.

analysed 10µl from each O/N RT/PCR RXN also 2µl of purified 1.5Kb alongside the 1Kb ladder. (looking for a 2.25K fragment)

Cloroform extracted RT/PCR products and EtOH ppt'd along with

V good - looks as though both nucleocapsid preps are yielding good genomic template and there is abundant 2.25Kb RT/PCR product Bam/Sph.

Will proceed with other RT/PCR RXNs as soon as possible - next week sometime (possibly Wed.)

To Page No.____

Witnessed & Understood by me, Date
Invented by Date
Recorded by David K. Clarke

TITLE: Transformation of bacteria with ~~2.25Kb~~ 1.5Kb frag.

Project No.

Book No.

From Page No.

Prepared 300ml L-Agar - autoclaved

Infected Hep-2 cells with P_3V (each 75cm² flask with 0.75ml virus)

Also infected Veros with V_6DC5-1 (each 25cm² flask with 0.25ml)

adsorbed ≈ 3hrs at 37°C

Prepared a 1% agarose gel for purification of 2.25Kb fragment.

Carried out bacterial transformation with ligation mixture containing 1.5Kb fragment. left on ice for 1hr then heat shocked at 42°C for 30secs prior to incubation in SOC for phenotypic expression (1hr 37°C).

Spun down 2.25Kb PCR product - combined with V_3 and V_3H_1 DNA and run out on a 1% Agarose gel - purified onto NA45 paper and then eluted - Phenol Choloform extracted - ppted with 3µl of glycogen. - There appeared to be plenty of DNA in the 2.25Kb band (should have ~1µg after purificat^n.)

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by

Project No.
Book No.
TITLE RT/PCR For Xca/Bam and Hha/Xca.

From Page No.

Checked bacterial transformants - looks good; there are the 'right' number.

Checked 293 cells - they are not ready - will try setting up some myself O/N for use tomorrow.

Checked c.p.e. on Vero cells infected with B9320 - beginnings of c.p.e apparent (more so at 37°C as expected and more at undil vs dil). Will check again this afternoon.

Hep-2 infected with V_3 - some c.p.e apparent - cells were initially only 60-70% confluent and may not yield as much virus - will check again this afternoon.

Set up some fresh 293 cells - Took a TC 150 and trypsinised into 2ml of Tryp-EDTA. Added 1ml to 18ml medium and plated out into 6 wells of a 6well plate (3ml/well). Similarly added 0.5ml cell sus. to 6ml of MEM and plated into 2 wells, and finally added 0.1ml cell suspension to 6ml MEM and plated out (↑↓ to shake plates) 37°C O/N.

Initiated RT/PCR Rxns for Xca to Bam (2.68K frag.) and Hha to Xca (2.28K frag.)

Set up Rev. transcriptions as before - let run for ≈1.5hrs

Then proceed to heat inactivate and set up PCR

Meanwhile set up O/N mini preps x10 in Amp$^+$ L-B

To Page No.

Witnessed & Understood by me, Syr W. Kelk | Date | Invented by | Date

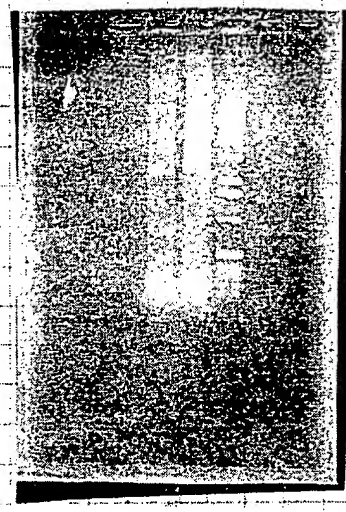
Recorded by David K. Clarke.

000261

Project No.
TITLE B9320 Rescue / 1.5K mini preps analysis. Book No.

From Page No.

Ran 1% Agarose gel to analyse RT/PCR products for Xcu/Bam and Hha/Xca



V. good – both products are as expected and in fairly good abundance.

500ng lesser band (total prod)
1μg greater band (total prod).

Cloroform extracted and EtOH ppt^ed with 4μl glycogen. – Can gel purify tomorrow.

Carried out mini preps in search of potential 1.5K clone (sph/Pml)
~~(Still need to do Bam/sph synthesis – will do when doing 3' fragment)~~
Did EcoRI digestion to check 1.5K insert. – ran 2 mini gels to check for insert.

Carried out transfection of B9320 (V_6 DC5, 32°c) infected (1ml ≈1.5hrs) 293 cells (O/N cell culture)
Used ≈ 12μl of In Vitro transcribed RNA (some from earlier IVT^n ≈6μl) for transfection.
left transfection mixture on for 4.5hrs at 37°c – then replaced with 1ml MEM – left at 37°c O/N.

Meanwhile harvested V_3H_1 32°c ATCC A2 virus for working stock. Also harvested B9320 Dl/undil at 32°/37°.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.

Book No.

TITLE

From Page No. 23

Continued from other page

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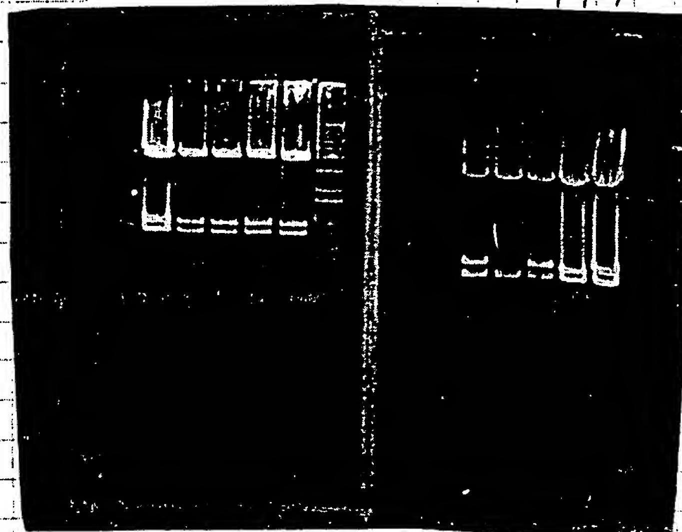


Good gel shows both 1.5 and 1.6K inserts with central EcoRI as expected; however will recut clones ①, ② and ③ to check insert size more carefully.

It looks as though ② and ③ represent both predicted bands.

Will cut with BamHI on one side and Not I or XhoI on the other but will keep entire clone bank for the moment.

Size difference in 924 bp fragment between 1.5K and 1.6K clones indicates that the size difference is generated at the SphI priming site.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

From Page No.

Prepared 1% Agarose gel for purification of Xca/Bam and Hha/Xca RT/PCR products.

Checked infected/transfected 293 cells - they look pretty sickly and there is evidence of c.p.e. - will harvest cell extracts

Ordered BstUI to HhaI and HhaI to BstUI

Proceeded to elute RT/PCR products from the gel and then phenol chloroform extracted and EtOH ppt^ed with glycogen

Will analyse all three purified DNAs (1/3 of each prep) on a 1% agarose gel over the weekend

Extracted cytoplasmic contents from transfected 293 cells - froze in EtOH/Dry ice and stored at -80°C

Will assay on Sat or Sun

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K Clarke

Project No.
Book No.
TITLE S.A.B. Meeting / RSV presentation

From Page No.

Gave RSV presentation to S.A.B 10-45am

Carried out Bam HI digestion on 1, 2, 3, 8 (mini prep DNA samples) to further characterise DNA insert size – gave ~1.5hrs at 37°C then phenol chloroform extracted and EtOH pptd.

Meanwhile carried out $TCID_{50s}$ on latest R9320 populations

1) a). V_6 DCS, V_1 37°C, Undil.
b) V_6 DCS, V_1 37°C Dil.

2) a) V_6 DCS, V_1 32°C Undil.
b) V_6 DCS, V_1 32°C Dil.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

TITLE: CAT assay of 'B' Rescue

Project No.____
Book No.____

From Page No.__

Set up XhoI digests on 1.5K mini prep DNAs (1,2,3,8) which had been previously cut with Bam HI - Cut for ~1.5hrs and then froze away prior to gel analysis tomorrow

Also set up CAT assay using 20μl of 'B' extract - let run for ≥ 3hrs, then froze at -80°C prior to TLC analysis on Mon.

To Page No.____

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Quantitation of Purified RT/PCR products
Gel analysis of Bam/Xho cut Minis (1.5K)

From Page No.

prepared 1% agarose gel to analyse Bam/Xho cut Mini prep DNAs (for identification of correct cDNA clone) - also ran 1/3 of each gel purified RT/PCR product from further along the genome, to quantitate prior to setting up ligations.

* No 2 is the correct clone for the 1.5K insert. - will look for others at a later date as back-up.

2.25K band contains ≈ 20ng of DNA ≡ 30ng μl⁻¹ - will use 2μl for ligation.

Xcu to Bam band contains ≈ 10ng will need to use ≈ 10-12μl for ligation - ditto Hhu to Xcu.

proceeded to set up ligations with appropriate amounts of Vector + insert.

To Page No.

Witnessed & Understood by me. Date

Invented by Date

Recorded by David K Clarke

Project No.
TITLE: Transformation with 2.25K, Xca/Bam, Hha/Xca
Book No.

From Page No.

Ethyl Acetate extracted CAT assay material and dried down in the speed Vac.

≈ 11:15am Equilibrated the TLC tank ~2hrs prior to running the TLC

Meanwhile prepared 400ml L-Agar for transformations. – poured plates – dried, and added X-gal (30µl of 40mg ml^{-1})

Set up transformations using 5µl from each O/N ligation mixture – left on ice for 1hr → 42°C heat shock for 30secs → 1hr phenotypic expression at 37°C → plated out on X-gal^{+} (30µl of 40mg ml^{-1})

Took off TLC – dried and exposed O/N on X-ray film. It seemed good (as if there was good acetylation)

Diluted down "BstUI to Hha" and "Hha to BstUI" to 100ng µl^{-1} and stored as working stock at -20°C Stored remainder at -80°C

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.
Book No.
TITLE TLC of 'B' Rescue / O/N Minis for RT/PCR clones

From Page No.

Checked bacterial transformants - looks encouraging - there are many colonies, quite a few Blues but also many whites.
Meanwhile spun down 0.75ml of genomic RNA (x2) - oried - dried -> -20°C in preparation for RT/PCR.

Checked $TCID_{50}$s (will make a final reading on Thurs) - it appears that 32°C is better for B9320 in Veros than 37°C - will await new freshly broken out' Veros before attempting to further improve titer for this virus (will use a range of the highest titer stocks for this virus at that time)

Developed TLC for B rescue - looks a little confusing - will need to repeat for aesthetics - Set up 3 CAT assays - using 1μl, 5μl and 10μl of Cell extract - let run ~3hrs, then freeze away at -80°C until tomorrow when will do the TLC.

Meanwhile set up RT/PCR RXN: using BstUI to make cDNA -

Prepared 10ml of RNase A stock - 10mg ml⁻¹ - froze away at -20°C.

To Page No.

Witnessed & Understood by me, Sage W. Kibler | Date | Invented by | Date

Recorded by David K Clarke

TITLE Mini Preps of Bt. RT/PCR products

Project No.

Book No.

From Page No.

Checked growth of mini preps – Xcu/Bam are not growing as well (just as it seemed on the cloning plates – where the colonies were much smaller

Meanwhile prepared 1% Agarose gel to analyse RT/PCR products (BstUI to Hhu)

Also Ethyl acetate extracted 'B' CAT assays – and equilibrated TLC tank prior to running three samples – Ran TLC and exposed O/N

Meanwhile carried out Mini prep DNA extractions on 8 of from each group of Clones (2.25K, X-B, H-X)

Didn't grow well.

analysed all of extract at once.

Good, the 3.6K RT/PCR products are there – Probably ≈ 5Wng in total preps – will gel purify tomorrow

Run half of 2.25K, and H/X

See gel overpage

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

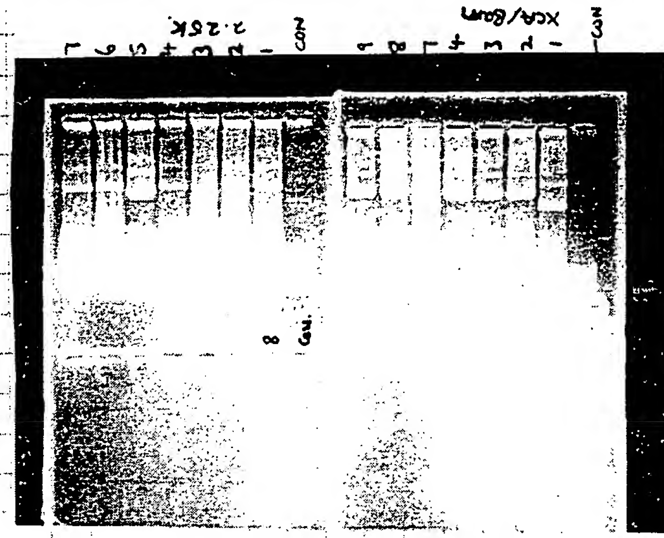
Recorded by David K Clarke

Project No.

Book No.

TITLE

From Page No. 31



Xca/Bam look good in spite of poor growth - 2, 4, 8, 9

2.25K also look good 1, 2, 3, 4 - 6, 7, 8 all look +ve

Can't read the Hha/Xca minis will rerun with control tomorrow

Will set up minis 2, 3, 4, 8 for X/B tomorrow (early - poor growers?)

1, 2, 3, 4 2.25K

To Page No.

Witnessed & Understood by me, Sergei W. Kohl

Date

Invented by

Date

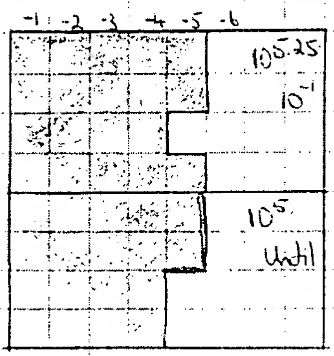
Recorded by David K Clarke

TITLE Gel purification of Bctu - Hhu   Project No. ____ Book No. ____

From Page No.__

Prepared 1% mini gel for analysis of H/X mini preps

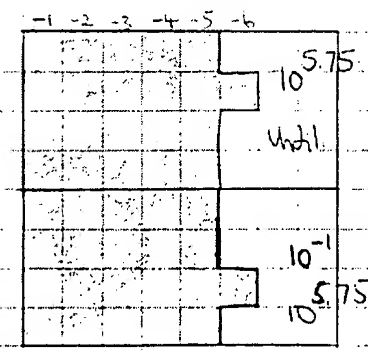
Did final reading of $TCID_{50s}$



$V_6 DCS_1 V_1$
B9320
37°C

Results of $TCID_{50s}$ indicate that 32°C is the best temp for growth of B9320

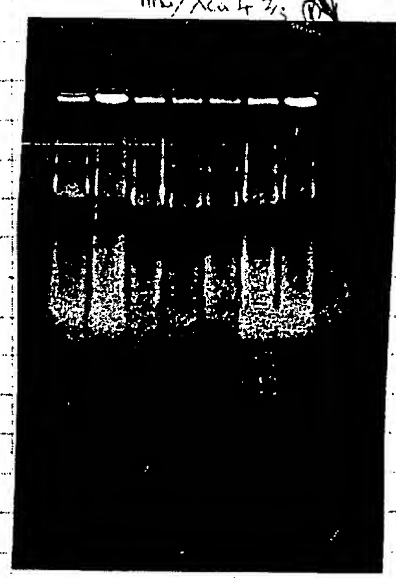
Will wait for 'New' veros before attempting to further boost titers. (will use highest titered stocks available to try and boost



$V_6 DCS_1 V_1$
B9320
32°C

8 7 6 5

Hhu/Xcu 4 3 2 1 con



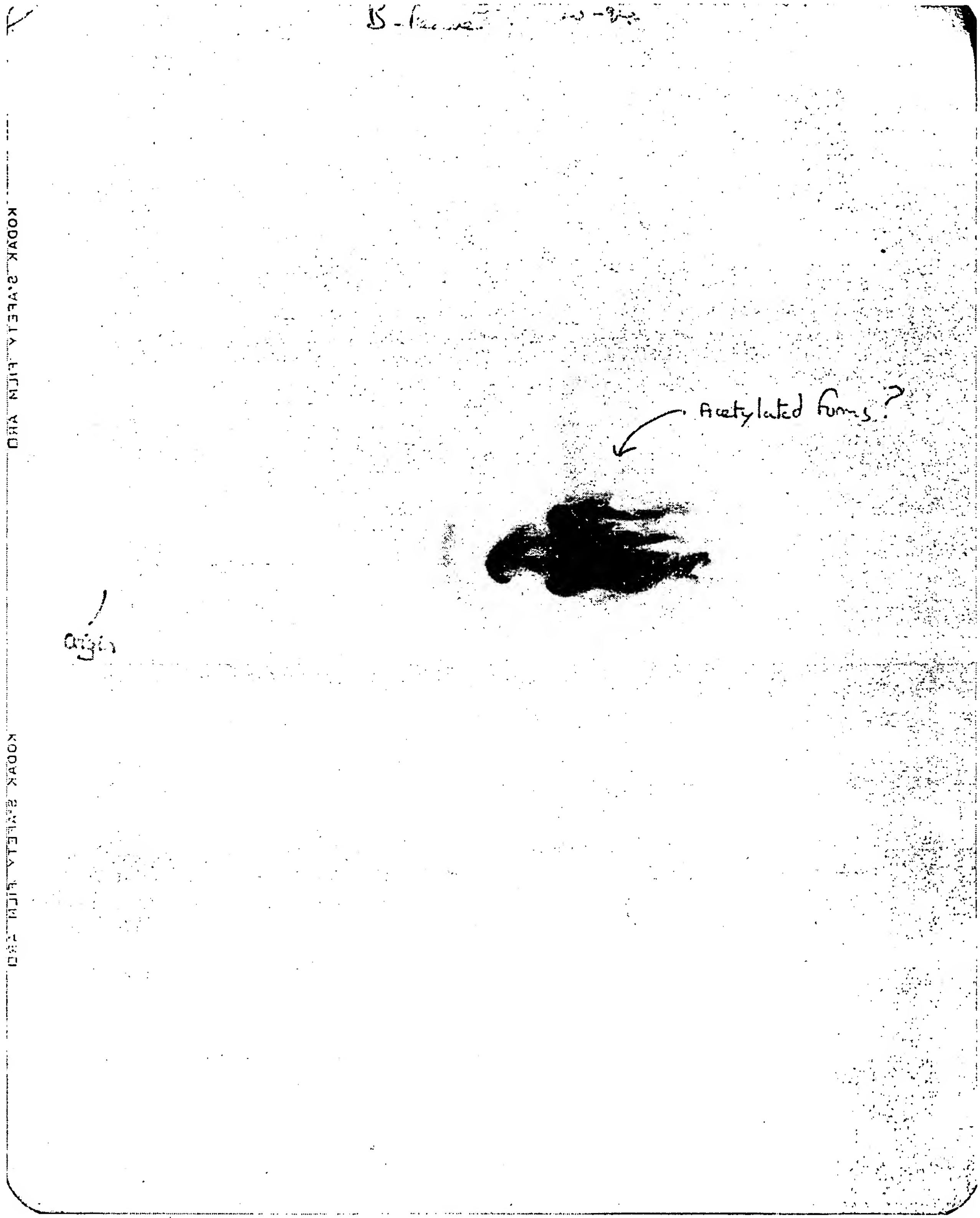
Unfortunately doesn't seem there are any clones containing the correct inserts, although will check 1, 7, and 8. - Will also look at 10 more clones

It is possible that the fainter lead band in the 'con' is the ccc form in which case 1, 7, and 8 would be correct (2.3K insert)

To Page No.____

Witnessed & Understood by me, Serge W. Kohl | Date | Invented by | Recorded by David K Clarke

B000272


Acetylated forms?
origin

Project No.____
Book No.____ TITLE ____

From Page No.__

Gel purified 36Kb BstEII to Hha fragment – phenol chloroform extracted and ethanol ppt'ed with glycogen

Set up the following O/N mini preps

Xca/Bam 2, 4
2.25K frag 2, 4 } for digestion
Hha / Xca 1, 8

Also set up 11-18 for uncut analysis Hha/Xca

Developed 'B' Rescue Cut Assay – it looks very good. It appears to be ≥ 20 fold better (see autorad)

To Page No.____

Witnessed & Understood by me, Sug W. Kibbe | Date | Invented by | Date

Recorded by David K Clarke

TITLE Digest^n Analysis of Potential 2.25K, Xca/Bam, and Hha/Xca Clones   Project No.   Book No.

From Page No.

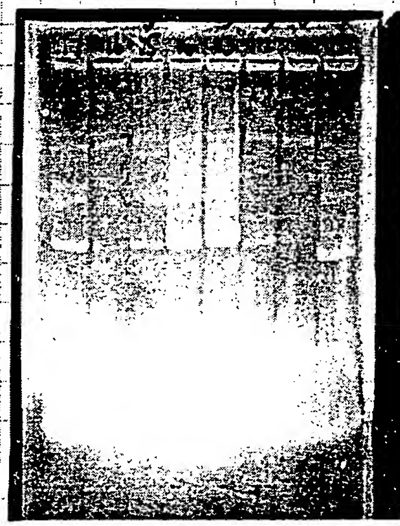
Carried out Mini prep DNA extractions on clones 11–18 for Hha/Xca to identify further clones with inserts

Kpn I / Xho I cut 2.25K Clones

Kpn I / Xho I cut Xca to Bam Clones (2.6K)

Kpn I / Not I cut Hha to Xca clones (2.3K)

Saved ½ of mini prep material for further digestions

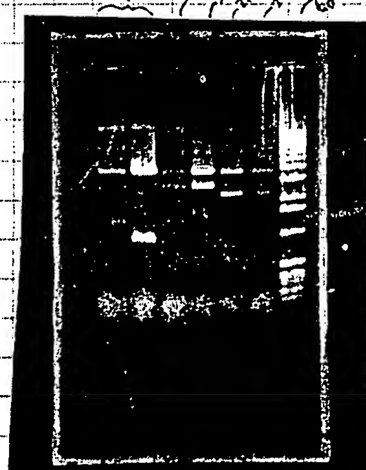


H/X clones 11, 12, 13 and 15 all look positive.

Also did double digests on 2.25, X/B, and H/X clones identified as positives yesterday

Meanwhile prepared L-Agar plates (Amp+) to restreak clone banks (Tomorrow)

Hha/Xca Xca-Bam Xca-Bam 2.25K 2.25K Std



looks as though the H/Xca inserts are not correct – will try 11, 12, and 13 with KpnI/NotI – will check some more for presence of insert

Both the 2.25K and Xca/Bam inserts look good – correct size as predicted

To Page No.

Witnessed & Understood by me, [signature]   Date

Invented by   Date

Recorded by David K Clarke

Project No.
Book No.
TITLE RT/PCR of Hha/Xca frag (2.3K) once again

From Page No.

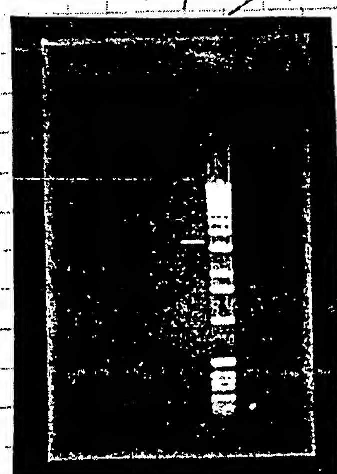
Checked PCR II Vector for F1 Ori - it's there - so will be able to use that plasmid for the site directed mutagenesis.

prepared 1% agarose gel for analysis of purified 3.6K fragment - also set up RT/PCR x 2 on Hha/Xca fragment - modified the annealing temp to 45°C for one RXN, left at 37°C for the other.

Will cut Xca/Bam Clone with EcoRI/PstI to give Vector + 1500bp, 700bp, 400bp

Will cut 2.25K clone with EcoRI/HinDIII to give Vector + ~980bp, ~1200bp

Restreaked Clone
Banks 3K, 1.5K, CAT/RSV
put at 37°C O/N.



Good the 3.5K frag looks fine and should have ~50ng total remaining. Will ligate that into 2µl of Vector.

To Page No.

Witnessed & Understood by me, Date
Invented by Date
Recorded by David K Clarke

TITLE: Analysis of ~~H~~/Xcu RT/PCR prods / O/N ligation of BstUI/Hha into PCRII Project No.____ Book No.____ 37

From Page No.__

Prepared 1% Agarose gel for analysis of RT/PCR products
Chloroform extracted and EtOH ppt^ed with glycogen

Set up O/N mini preps 11, 12, 13 H/X for digestion (Kpn/Not I)
Also set up 7 more minis for preliminary test.

Set up O/N ligations - All of remaining BstU-Xcu fragment with
2µl (50ng) of Vector in 20µl vol. 12°C o/n Also
set up a No insert control

Qualities required for 'L'-end Oligos

① T7 promoter Flush with precise end of RSV genome - get from CAT-RSV construct.

② Should contain 2-5 restriction sites to facilitate modification and/or cloning.

③

Qualities required for 'N'-end Oligos

① Should give precise run off transcripts ∴ include HgA I
② Should include 2-5 restriction sites not in genome to facilitate further modification and/or movement between vectors.

PCR up small fragment at L end - using ScaI / SphI / XmnI site(s) + end oligos - ligate to synthesised ds DNA containing promoter + restriction sites and a 1 base 5' overhang (T) - then end clone - use ~~HindIII~~ site ~~at end of cloned restriction sites to clone~~ back into PCR II containing Not I 3K clone.

To Page No.____

Witnessed & Understood by me, Date | Invented by | Date

Recorded by

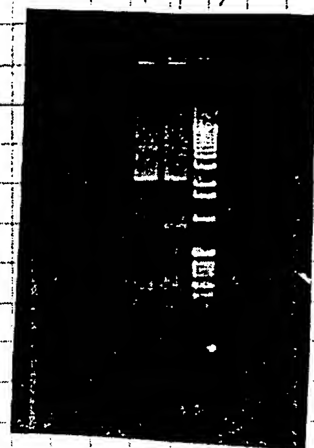
Project No.

Book No.

TITLE

From Page No. 37

45°C 37°C 1kb lad



Good Hha/Xcu RT/PCR products contain ~ 20ng for both samples = Total = 200ng

The 45°C annealing temp looks better than the 37°C, with a lot less low m.w. non specific product.

Will combine both and electro elute purify on tues.

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

Project No.

TITLE Transformation with BstXI/Hha Book No.

From Page No.

Carried out mini preps on Hha/Xca 11, 12, 13, 18, 19, 20, 21, 22, 23, 24

↓

KpnI/NotI digested

Did bacterial transformations with 5μl of O/N ligations mixtures → O/N 37°C

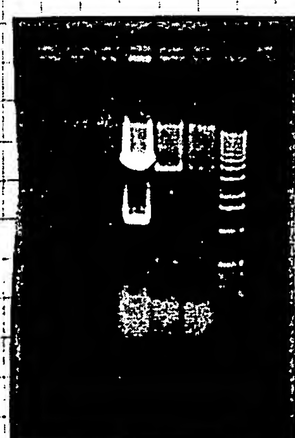
24 23 22 21 20 19 18 ladd

19 and 22 look like the ones that I want – will extract more DNA for Digestion later today

↓

Proceeded with extraction and phenol Chloroform extraction – left in EtOH until tomorrow (will then do KpnI/NotI/RNase digestions

Completed double digests on 11, 12, 13 and analysed the products on a 1% gel



No – there are no clones containing inserts of the correct size

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

[signature]

Recorded by [signature]

Project No.______
Book No.______

TITLE Double digestion of Potential Xca/Hha clones

From Page No.__

Checked clones – looks v. good – control plate is showing predominantly Blue colonies, while BstH/Hha are showing predominantly White colonies.

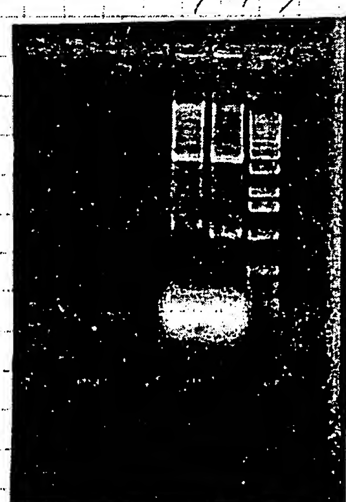
Set up restriction analysis on H/X 22 and 19. KpnI/Not I

prepared 1% Agarose gel for analysis of digestion products

Also prepared a 1% gel for purification of Hha/Xca fragment.

Set up o/n minis of potential Bst-Hha clones – and also streaked them onto master plates.

22 H/X 19 H/X ladder



Completed purification of 'New' Hha/Xca fragment

Phenol Chloroform extracted and EtOH ppted with glycogen.

19 and 22 are not the correct clones either – both are ~1000 bases shorter than what I am looking for.

Will look at single digest products next time, to make sure there are no internal KpnI or Not I sites.

To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Khle		Recorded by David K' Clarke	

TITLE Mini preps on Potential BstUI/Hha Clones

Project No.

Book No.

From Page No.

Carried out mini preps on BstUI/Hha clones 1-7

loaded 1/4 on a gel for a relative comparison (oops forgot no-insert control)

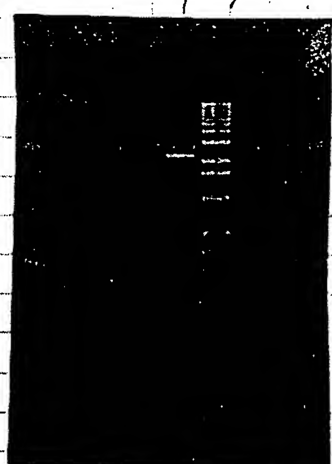
Also digested 3/4 of each with EcoRI/Rnase A ~1.5-2hrs at 37°C

Also checked purified Hha/Xca fragment on a gel to check conc etc

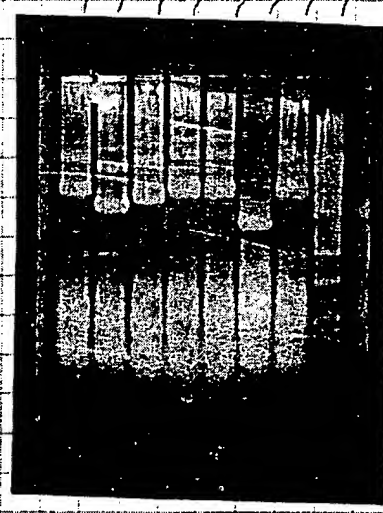
looked at 1/5 (2µl out of 10)

Hha/Xca

1Kb Ladder



7 6 5 4 3 2 1 lad



1, 3, 4, 7 look as though they could be positives

Will check their EcoRI restriction pattern

Also checked yield from Hha/Xca purification - looks pretty good and fairly pure - Used 4µl in ligation with 2µl Vector - 12°C ON

Prepared yet another 1% Agarose gel to analyse EcoRI digests - they look good (see overpage)

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

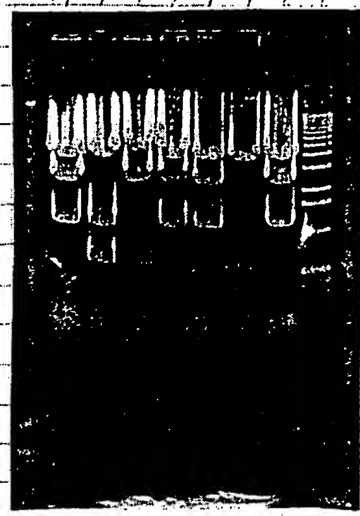
Date

Recorded by Ward K Clarke

Project No._______
Book No._______ TITLE __________

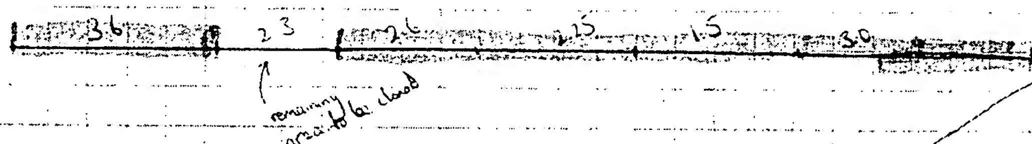
From Page No.__

7* 6 5 4* 3* 2 1* λHB std



1, 3, 4, 7 are indeed correct, good! now have all but the 2.3K frag cloned (see below).

prepared a fresh stock of Amp 50mg/ml



To Page No.____

Witnessed & Understood by me, [signature] Date

Invented by Date

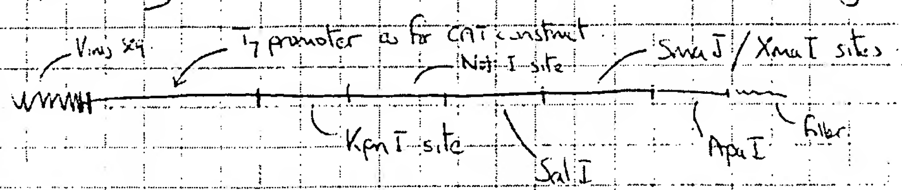
Recorded by [signature]

TITLE ______ Project No. ______ Book No. ______

From Page No. __

Prepared L-Agar for transformation

Prepared Oligos for modification of the Virus' 5' end (L-gene)



5' TAT [redacted] GAG [redacted] TAT [redacted] GTA CC[redacted] CGG [redacted] C GT[redacted]

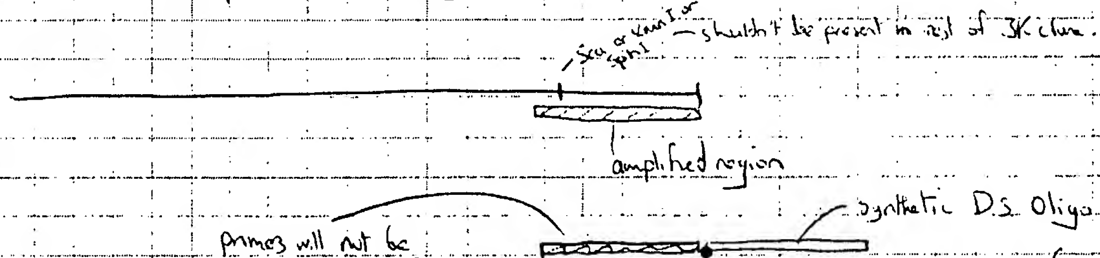
CCC [redacted] CC[redacted] CA[redacted] 3' 52-mer

Complementary Oligo

5' [redacted] TGG [redacted] GGC [redacted] GG [redacted] ACG [redacted] CCG

[redacted] TAC [redacted] ATA [redacted] CTC [redacted] ATA 3'

Will PCR up a small fragment of the end portion of the L-gene



primers will not be Kinased ∴ no 5'P so will not self ligate.

Will then ligate both pieces of DNA together (only one of the synthetic oligos will be Kinased) and MM with restriction enzymes and reinsert into 3K clone appropriately cut to accept the frag

need to check 3K clone orientation with initial double digest

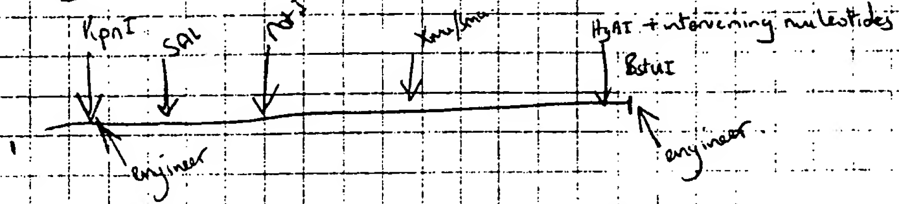
To Page No. ____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

Project No.
Book No. TITLE

From Page No. 43

For the 3' end of the Virus will use synthetic Oligos, with an engineered BstUI, HgAI and KpnI, NotI, SalI, Xma/Sma



Need to check orientation of 3.6K clone(s) to find one with the correctly positioned (on the ~~right~~ correct side)

Will ligate synthetic DNA into BstUI/KpnI cut construct (Syn DNA Will lack 5' phosphates so will not self ligate)

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

TITLE O/N Minis/masters for Potential 23K Clones **Project No.** ____ **Book No.** ____

From Page No. ____

Checked bacterial transformants - there are significantly more whites vs control with the 23K RT/PCR product - let grow for another few hours - then set up O/N minis (will wait to set up masters from the O/N cultures) - Will Do 14 initially.

Designed Oligos for 3' end of genome construct

Filler KpN I, KpNI cut, Sal I, Not I, Xma/Sma, HgA I

Will engineer KpnI site: 5' ~~TCGGAC~~ [redacted] C GTC GAC GCGGCCGC GC C GCGGCCG GCG GAT

cuts, BstHI cuts: TT ACG

~~39 mer~~ 34mer

The complement

5'- CGT AAT ATGG GGT CCC CGG GGC GGC CGC GTC GAC GGT AC[redacted] KpnI cut

38mer

[redacted] = sequence omitted from oligos to accommodate KpnI ends

Xca to Bam clone will contain the non-translated region of 'F' where a mutation can be introduced to give a restriction site.

(PmeI) can be introduced at ~ position 7420 (see Book) will sequence around this region from the translated portion to verify sequence.
Will need to do large scale preps of Xca/Bam to get plasmid for sequencing

To Page No. ____

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

From Page No. 45

Can also introduce a StuI site AGG↓CCT in the hydrophobic Anchor domain of the F' protein without altering the coding capacity at position ~ 7292

$\underbrace{GTT}_{Val}\ \underbrace{GGA}_{Gly}$ orig

$\underbrace{GTA}_{Val}\ \underbrace{GGC}_{Gly}\ \underbrace{ctg}_{leu}$

Set up 14 O/N minis — will extract DNA tomorrow and do a preliminary gel analysis.

To Page No.

Witnessed & Understood by me: Serg W. Kohle | Date | Invented by | Date

Recorded by David K Clarke

Project No.

TITLE

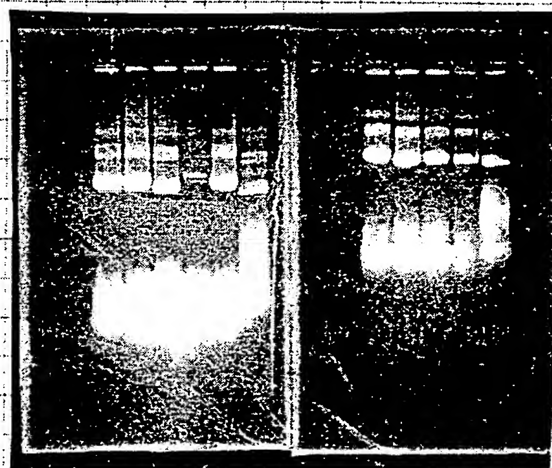
Book No.

From Page No.

Went through mini preps in search of 2.3K Hha/Xca clone
≈ 5 of the mini preps either hadn't grown at all, or only very weakly
Extracted DNA from the other 7

Prepared 2 x 1% Agarose gels and ran out minis with PCR II control plasmid.

However - Will try another 14 mini preps just to make sure



It doesn't look as though there are any clones of the correct size

This indicates that either the SH, or G protein are toxic - will have to devise a new scheme to clone this area

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

Project No.____

Book No.____

TITLE PCR reamplification of Hha/Xca DNA.

From Page No.__

Set up ten more mini preps to check for potential 2.3K (Hha/Xca) clones.

Also set up PCR reaction on remaining purified Hha/Xca DNA

6µl $MgCl_2$
8µl x10 Rxn buffer
2µl each dNTP
2µl each primer
H_2O to ≈ 80µl
0.6µl Taq. 45°C annealing temp.
30 cycles.

There is a unique AvrII site at position 2129 (towards the end of the 'N' gene)

can use this to make cDNA → XhoI site at position 4481

May be able to make a cDNA clone, then from ~~PFET~~ XhoI to either PflMI or Eam1105I - depending on ....

To Page No.____

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by [signature]

TITLE Testing Mini Preps for 2.3K insert

Project No.

Book No.

From Page No.

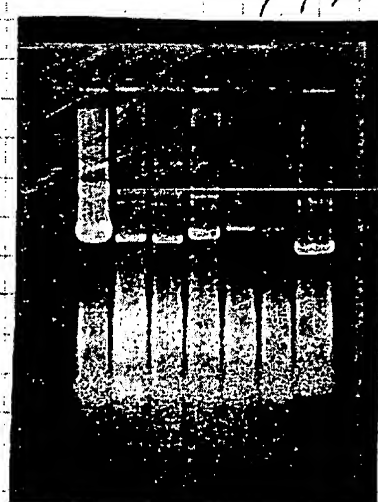
Run out a sample of Ilhu/X amplified DNA to assess amount of amplification - Not v. good - little amplification occurred. Tossed sample - will try RT/PCR using new primers etc

Invitrogen One shot host is EcoK$^{(-)}$ but Dam^{+}/Dcm^{+}

Will ∴ need to use Eam 1105 I - will check availability tomorrow

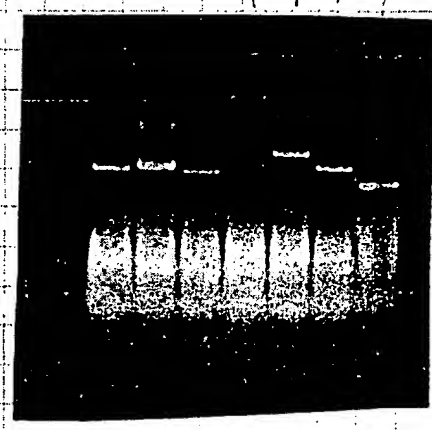
Meanwhile carried out mini prep DNA extractions on 12 more potential Ilhu/Xca clones - Run 1/4 of each prep on a 1% Agarose gel - froze the remaining 3/4 at -20°C

10 1 cut possibles



Will cut 1, 10, 25, 26, 27 with Kpn - look at 1/3 + Not I (RNase A)

27 26 25 1kb



To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by

Project No.

Book No. TITLE

From Page No.

Back to work!

Phenol-Cloroform extracted mini preps 1,10,25,26,27 and EtOH ppted

Then set up KpnI digestions — Gave 2 1hr at 37°C

Prepared 1% Agarose gel for analysis of 1st Cut.

To Page No.

Witnessed & Understood by me, Date

Sage W. White

Invented by Date

Recorded by David K Clarke.

TITLE B9320 infections – Analysis of potential 2.3K clones

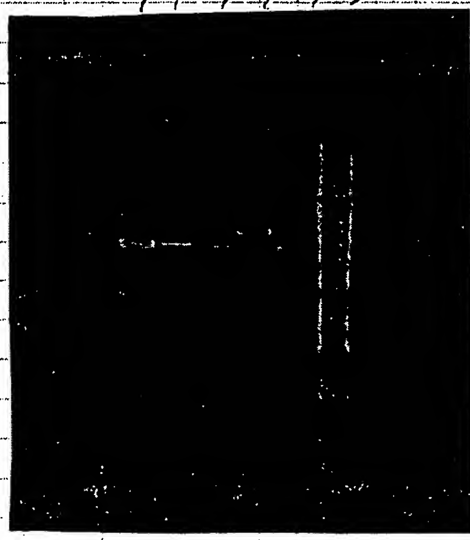
Project No.

Book No.

From Page No.

Set up 1% Agarose gel and analyzed KpnI digests of potential 2.3K clones (Phenol Chloroform extracted the bulk of the DNA and EtOH ppt'd prior to cutting with NotI)

27, 26, 25, 10, 1, ladder



Bst 11071 is an Isoschizomer of XCA I

It is clear that even though CCC plasmid migrate the same distance they may contain different sized inserts

10 looks as though it is the correct size ~6Kb to contain the 2.3K insert.

Infected Vero TC25s with a variety of B9320 stocks (to boost higher titer stocks)

① V_6 DC5, V_1 32°C → D.I / Undil at 32°C

② V_6 DC5, 32°C → D.I / Undil 32°C

③ V_5 Undil → D.I / Undil 32°C

Used 0.25 ml of inoculum for each TC25 and adsorbed for ~3hs at 37°C — then placed at 32°C

To Page No.

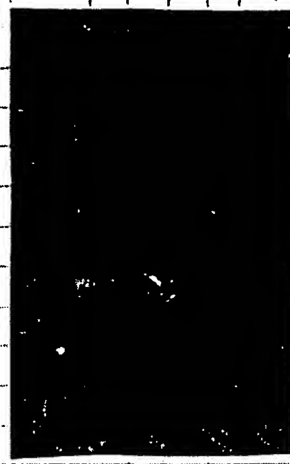
Witnessed & Understood by me, Date

Invented by Date

Recorded by

Project No.
Book No.
TITLE

From Page No.



Number 10 looks like it {should / could} be the one. The Insert size is correct 2.3Kb as is the regenerated

Will set up a fresh o/N culture to double check — will also streak out on a separate plate as a reserve

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kable		Recorded by David K Clarke	

TITLE: Checking Clone 10 for Hba/Xca Insert   Project No. ____ Book No. ____

From Page No. __

Carried out mini prep DNA extraction on clone 10 – froze down x3 aliquots of this O/N culture in 15% glycerol, to act as a −80°C stock.

Set up Bst 1107 I digestion on mini prep DNA in 40µl final vol; incubated at 37°C (unusual for B. stearothermophilus) for 1-2hrs – used 1µl of enzyme. Then phenol chloroform extracted and EtOH ppted.

Checked c.p.e. on infected Veros – very little sign of c.p.e., but will check again later today.

Proceeded to set up Sac I digestion – (while retaining a sample of Bst 1107 I cut DNA for gel analysis) – in 30µl final vol.; 37°C 1µl Enz.

NB Strategy for RSV vaccine production, using naked DNA.

1) Put the CMV promoter just downstream of the entire cloned RSV genome (cDNA) – maybe engineer in splice sites between genes to give monocistronic messages OR put a CMV promoter in the genome between each gene to give ~~a~~ individual mRNA synthesis from each promoter.

Ordered the 4 Oligos necessary for modification of the 3' and 5' ends of the RSV genome cDNA.

~~Rechecked cpe which are now becoming more apparent.~~

To Page No. ____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.

Book No.

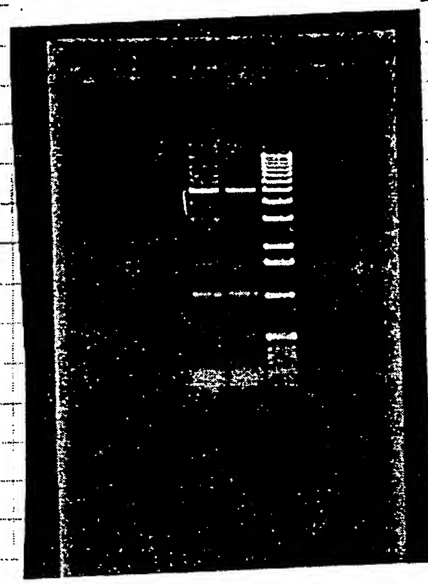
TITLE

From Page No.

Bst1107I + SacI

Bst1107I

ladder



Restriction pattern indicates that clone 10 is <u>NOT</u> what we want - I should <u>not</u> have 2 Bst1107I sites

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Gary W. Keble		Recorded by David K Clarke	

B 9320 Harvests.

TITLE: RT/PCR of Xho/Xca region of RSV.

Project No.______

Book No.______

From Page No.__

Prepared L-Agar for transformation later today (Need 30°C incubator)

Set up RT/PCR RXN using XhoI to Xca primer (XhoI to Bam) to try and split up the clone that's causing problems. Also ran a rxn for Pamuk to try and improve her cloning efficiency.

RT/PCR product should be 1.35Kb

Electroporated some E. coli (George's) with 1µl of ligated 2.3K fragment with PCR II (1.8KV)

Carried out phenotypic expression at 30°C to minimise recombination etc

3-40 to 4-40pm - plated out and put 2 at 30°C and 1 at 37°C

Harvested 'B'9320 virus infections and froze at -80°C - will run TCID50s on Monday

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

Project No.

Book No.

TITLE Analysis of RT/PCR products (Xho/Xcn)

From Page No.

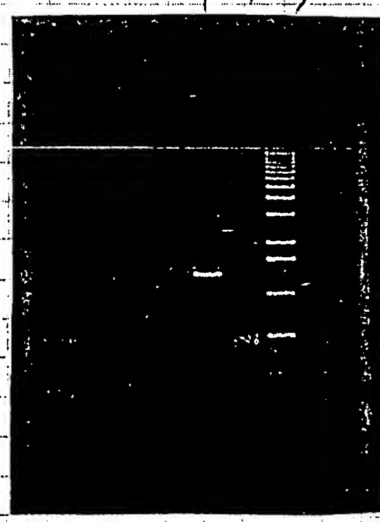
Prepared 1% Agarose gel for analysis of RT/PCR products – Ran out 1Kb ladder, Pannuk's RXN, and mine

Also checked electroporation clones – there are quite a few whites at 37°C – streaked out ~36 and grew o/n at 37°C as a master plate

Also checked clones incubated at 30°C – there are quite a few there too – the colonies are smaller and blue colour has not yet developed – may incubate for a further 24hrs or may plate out on a master plate later today.

Streaked out clones grown at 30°C → 30°C master plate

Will set up Minis tomorrow.



Good the Xho/Xcn frag looks perfect – at the correct position and in good abundance

Pannuk's RXN did not work.

To Page No.

Witnessed & Understood by me. | Date | Invented by | Date

Recorded by

O/N minis

TITLE: Gel purification of Xho/Xca RT/PCR product

Project No.

Book No.

From Page No.

Prepared 1% Agarose gel for purification of Xho/Xca RT/PCR products - run gel and proceeded with electroelution of the DNA Band - everything went well - Phenol/Cloroform extracted and EtOH ppt^ed with glycogen carrier - will set up O/N ligation tomorrow. Checked O/N growth of master plates - interestingly the clones grown at 30°C showed more uniform and stronger growth than those growing at 37°C - also on the original plates the lack of blue colour expression at 30°C (even following O/N at 4°C) indicates that there may be no expression from the lac-Z region of the transforming plasmid (this region contains the RSV cDNA insert).

To Page No.

Witnessed & Understood by me, Serge W. Kh[illegible]

Date

Invented by

Date

Recorded by David K Clarke

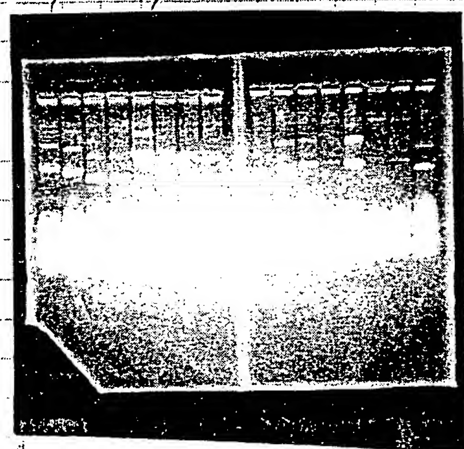
Project No.
Book No.
TITLE Mini prep Analysis of Bt. Hha/Xca clones

From Page No.

Carried out mini-prep DNA extractions on clones grown at 30° and 37°C - Prepared 2 x 1% Agarose gel for 1st look analysis

loaded 10μl (of 40) for each sample

10 6



Will take a look at Nos 6 and 10 but neither look v. promising

phenol chloroform extracted these samples and EtOH ppt'd - prior to digestion and gel analysis

Set up O/N ligations of purified Xho/Xca - used ⅓ of total purified material

also set up control ligation

Carried out TCID$_{50s}$ on V_6 32°C B9320

V_6DC5; V_1 B9320 32°

V_6DC5; V_2 B9320 32°

Set up O/N mini preps of the two 'end' clones 3Kb NCTA1 at 5' (Len)
3.6Kb Bstu/Hha at 3' (Nen)

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by

Transformation with Xho/Xca

TITLE: Digestion of Miniprep DNA - potential 2.3K Hha/Xca

Project No.

Book No.

From Page No.

Set up Bst1107I digestions on 6 and 10 from yesterday

prepared 1% agarose gel for analysis

Called BRL about plasmid/clone stability - they said HB101 performed best and to use rich broth (Terrific broth, SOC media/SOB agar) and not to grow cells past logarithmic phase

Called Invitrogen - They suggested using Top10 or DH10 Hosts! - not very knowledgeable

Called Clontech - Not much help - may get back to me

Called Promega - Rick X 1360 - suggested JM109 and recommended Stratagene (Sure-clone strains)

Completed double digest on 6 and 10 with Xho I - run on gel for analysis

Also carried out transformation with Xho/Xca RT/PCR product + Control

Replated Bstu/Hha bugs in a fresh agar plate to keep viable - grew O/N at 37°C

To Page No.

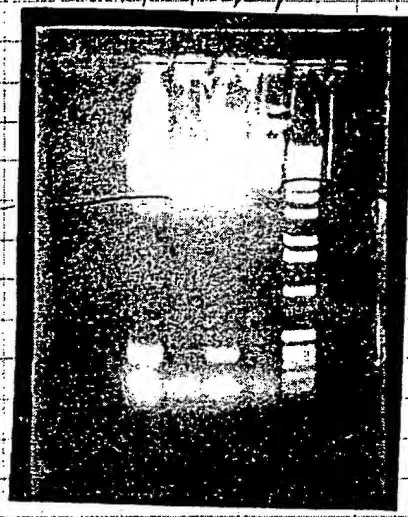
Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

Project No.____

Book No.____ TITLE ____

From Page No. 59

10Bst/Xho 10Bst 6Bst/Xho 6Bst



uncut plasmid

uncut plasmid

These clones are not correct either. (There are some sort of rearrangements going on)

Will wait and see what Xho/Xca clones bring.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kibble		Recorded by David K Clarke	

TITLE Large Plasmid Preps for 3.6K and 3K RSV clones

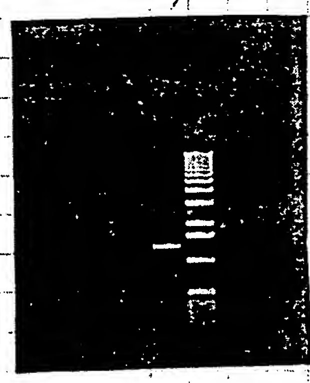
From Page No.

Checked transformants – not a bean – no white colonies – indicates G-protein is highly toxic – may be the problem!

Called Stratagene (Brian) – suggested using 'SURE'-strain which minimises recombination events and/or 'Able C' and 'Able K' – probably should not use Blue colour selection since it will induce expression from the lac 'Z' promoter / will reduce plasmid copy number/expression

Started plasmid preps – Qiagen

Purified Xho/Xca fragment



This gel proves that there was an abundance of DNA in the ligation with PCR II vector

∴ this region must indeed be very toxic to bacteria (it contains the entire coding sequence of the 'G' protein).

Things to try

1) Able C, K, Sure cells (strains) +/– blue selection +/– Rich medium.
2) RT/PCR Hha/Xca again and ligate to 3.6K clone and Xca/Bam appropriately cut – then clone large fragment
3) Clone fragment in Yeast
4) Cut Hha/Xca fragment with Xho – try cloning both fragments
5) Clone Hha/Xca into pBR322 → HB101 or other strain

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.________
Book No.________ TITLE Analysis of 3K and 3.6K RSV genomic clones in preparation for end modification.

From Page No.__

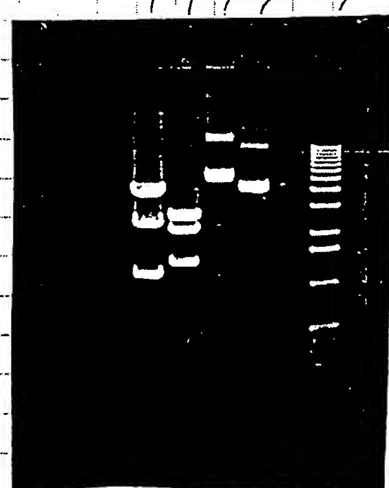
Will cut the 3K clone with Sca I (has 2 sites - internal) and one in the plasmid - 3 total

Will cut 3.6K clone with EcoRI (one internal site - two flanking sites)

Will also trim some PCR II Vector with Xho I and the small amount of remaining purified Hha/Xca fragment.

Prepared 1% Gel and run out digests

LHS. lad / Hha/Xca/Xho / Uncut 3K / Uncut 3.6K / Sca Cut 3K / Eco cut 3.6K)



There was not enough DNA to see on the Hha/Xca Xho digestion

Phenol chloroform extracted Xho cut PCR II - ppt^ed with glycogen

Good - plasmid preps look fine and the Sca and Eco sites are as expected

Wrong! 3K clone is unstable and has undergone deletion of part of the insert or plasmid

To Page No.____

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K Clarke

TITLE: Quantitation of Purified Oligos

Project No.
Book No.

From Page No.

It appears that the 3K clone has undergone a 1K deletion - need to go back and check minis from plates - if those are also deleted, then will have to transform from original minis into 'SURE' strains

Meanwhile dried down purified oligos from 50% ~~ethanol~~ methanol - resuspended in H_2O and measured O.D.

One of the Oligos had not been purified correctly - redissolved large white pellet in 3ml H_2O and loaded onto column - eluted - dried down

Stored dried oligos at -20° prior to dissolving in H_2O

Did a preliminary read on $TCID_{50s}$ - does not appear to be any stellar 'B' virus stocks - will check again on Tues for final read.

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

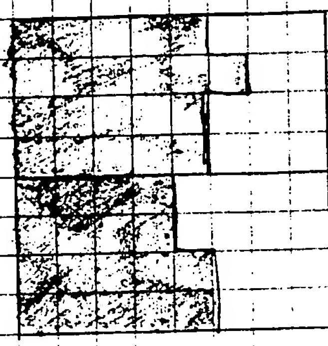
Project No.______
Book No.______

Back from San Diego weekend!!

TITLE Final $TCID_{50}$ Readings on B-virus stocks.

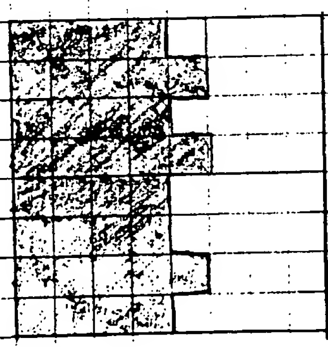
From Page No.__

Made final $TCID_{50}$ Readings on 'B'-virus stocks

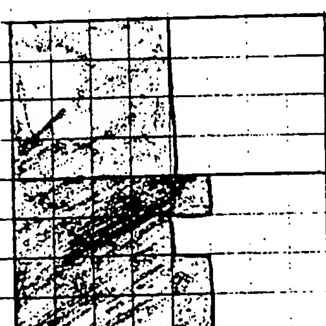


B9320 $V_6 DC5_1 V_1$
10^{-1} (32°C)

Undil



10^{-1}
B9320 $V_6 DC5_1 V_2$
(32°)
Undil



(10^{-1})
B9320 V_6 32°C

(Undil)

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Sey. W. Kble		Recorded by David K Clarke.	

TITLE: ~~Bact~~ Bacterial Transformation ('SURE', 'ABLE' cells)

Project No.

Book No.

From Page No.

prepared 300ml L-Agar for transformation of 'SURE' 'C-ABLE', 'K-ABLE' and 3K DNA

Redissolved purified Oligos in 200µl H_2O and measured the $O.D_{260}$ (used 10µl diluted into 490µl H_2O) - Yields look good 0.7 - 2µg µl⁻¹ for each

Set up the following transformations.

~3µl of Hha/Xca (2.3K) ligation mixture each into Able C, " K

~1µl of NE13 (3.3K) mini prep DNA into Able C, " K, Sure

} 100µl of cells for each Trans^n

1µl of 'CON' pUC18 in 50µl each of Able C', Able K, Sure

Set up O/N mini preps of 1.5K clones (2) only
2.25K clones ×2 (Bam/Pml?)
Xca/Bam Clones ×2.

Have 3.6K clone grown up - it is okay
Am transforming with 3K clone - so will not do O/N minis on that one.

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

[signature]

Recorded by David K Clarke

Project No.

Book No.

TITLE Analysis of 2.25K and Xcu/Bam clones by ligation.

From Page No.

Checked bacterial transformants (Sure - Able C - Able K) - Efficiencies with Able C' and 'K' are lower than with 'SURE' - however did get a few transformants on 'C' and 'K' with 2.3K (Hha/Xcu) ligat^n mixture - many more clones on 'Sure' - will set up ~24 mini preps O/N

Meanwhile did mini preps on 2.25K clones X/Bam clones - left 1.5K mini prep to grow longer - (1.5K did grow finally and carried out mini prep DNA extraction

Infected 10 x TC 150 each with 1ml of V_3H_1 virus stock - adsorbed ≈ 3hrs at 37°C, then added growth medium and incubated at 32°C

Set up Digestions on mini-prep DNAs XcuI/BamHI and 2.25K clone

1. Bam HI on each ~1.5hrs
2. Either SphI or Bst 1107 I. ~2hrs

Will look for 2.25K or 2.6K.

Proceeded to analyze the products on a 1% Agarose gel.

Good, both clones for each of 'Xcu/Bam' and '2.25K' clones contain the appropriate sized inserts (+ restriction sites)

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kble [signature]		Recorded by David K Clarke [signature]	

000306

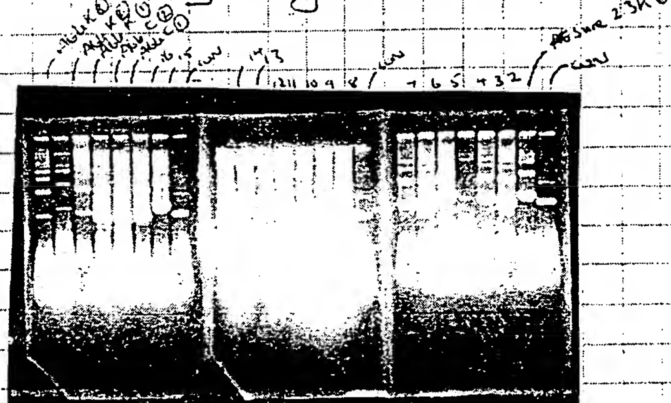
TITLE Mini prep analysis of potential 2.3K clones.

From Page No.__

Prepared L-Agar - Amp plates to restreak clone banks.

Then proceeded with mini preps for potential 2.3K clones in 'Sure' 'Able' etc.

prepared 3 x 1% Agarose gels for analysis.



Will take a look at Able K' ② by double digestion, but unfortunately it doesn't look as though any 2.3K clones were obtained - even with the 'Sure' - Able C/K' systems.

Restreaked entire clone bank - will check again tomorrow and store at 4°C if they are fully grown.

① Use HB101 / pBR322 to clone this fragment.

② Use yeast

③ ligate the RT/PCR product to the cloned DNA on either side and then try cloning.

Hep-2 cells are showing nice c.p.e. already at 24h.p.i. Will harvest as early as possible tomorrow morning.

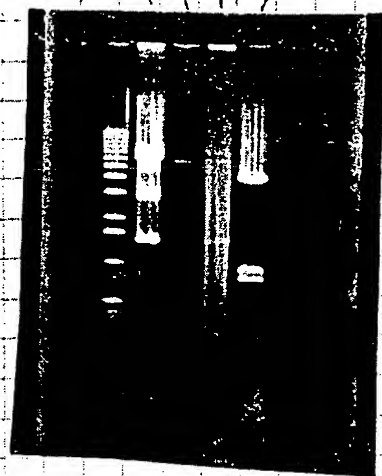
To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Ajy W. Kble		Recorded by David K Clarke	

Project No.______
Book No.______ TITLE ______

From Page No.__

3K Sca Cut in 'Sure'
3K Sca Cut in pBlue K
3K Sca Cut in pBlue C
1.5K EcoCut (looks okay)



Need to check
3K 'Sure' with EcoRI and
Bam HI

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kleble		Recorded by David K Clarke	

Project No.

TITLE Nucleocapsid Preparation Book No.

From Page No.

Cpe on infected Heps was v. extensive - initiated Nucleocapsid prep at 9am - let the Nucleocapsid pellet resuspend on ice prior to loading onto K' tartrate gradients.

Run only 2 gradients this time.

Also carried out minipreps on remaining SURE clones (forgot control on gel)

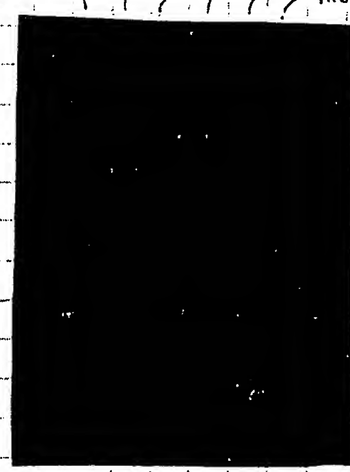
Run on a 1% Agarose gel

Carried out digestion of "3K in Sure" (BamHI and EcoRI separately)

Also did an EcoRI on AbleK ② (Page 67)

All 'SURE' mini preps showed plasmid of the same size - Didn't have 'CON' so can't tell if there is an insert - re ran one sample with 'CON' to check again

SURE 2.3 RRO cut Eco AbleK② BamCut 3K Sure EcoCut 3K Sure 1Kb ladder



Will streak out '3K SURE' on an Amp[+] plate to have a stock.

The insert size of both 'AbleK②' and Sure 2.3 is 2.3 too small

3K 'SURE' does appear to contain the correct insert (after EcoRI digest). However the correct fragments are running slightly faster / further than anticipated - this may be due to the fact that the digests were loaded directly onto the gel without dilution etc in electrophoresis buffer

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Nucleocapsid Harvest from gradients

From Page No.

Took off nucleocapsid spin - bands look good on both gradients - Harvested into 4 eppys - diluted and spin down in microfuge. Rinsed with T.E., then resuspended each pellet in 500µl TE, 5µl of stock Prot K, and 5µl of 20% SDS - Incubated at 37°C for ~45mins, then phenol chloroform extracted x2 - EtOH pptd - yield looks pretty good.

Infected Vero cells with BA320 V/DC5V, 10^{-1} 32°C - DA/undil at 32/37°C

Absorbed ~3hrs - then added G. medium.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

TITLE Transformation of HB101 with Hha/Xca DNA

Project No. ______

Book No. ______

From Page No.___

Called Jericho security!

Prepared fresh L-B Agar Amp$^+$ for transformation (42°C waterbath)

Set up transformation with 5µl of DNA from CW (pBR322) and my Hha/Xca ligation mixture.

Also set up Bam HI digest on 3K SURE - left ~1hr at 37°C

Then phenol chloroform extracted - ethanol ppt^{ed} in preparation for Xho I digestion.

Checked c.p.e in Veros infected with B9320 - 37°C show some c.p.e but little is visible at 32°C (will check again later today).

Designed Bam HI linkers

5' NNNNNGGATCC T ——— A [redacted] 3'

3' [redacted] A ——— T CCTAGGNNNNN 5'

[redacted] These Oligos are Kinased

Set up Xho I digest ~ let run for ~ 1hr - prepared a 1% Agarose gel and run to analyse digestion products

Bam/Xho cut '3K SURE'

ladder

[redacted]

Good. Insert look believeably to be 2.97Kb as predicted and expected (But may be a small deletion in the Vector.

Will try a larger scale prep soon.

3K Sure is okay

To Page No.___

Witnessed & Understood by me,	Date	Invented by	Date
Angus W. Kable (signature)			
		Recorded by David K. Clarke (signature)	

Project No.______

Book No.______ TITLE B9320 Harvests — O/N minis of potential Hha/Xca

From Page No.__

Checked transformants — there are plenty on the Xca/Hha plates — will select 14 for mini preps.

Checked c.p.e on B9320-infected Vero cells — They are v advanced at 37°C undil (complete fusion of monolayer) — less c.p.e at undil 37°C and still less at 32°C Dil/undil

Ordered the Bam HI linkers (from overpage) — linker (2) should be kinased

7 x 4 = 28°C
4 x 2 = 8
36°C Melting temp = 36°C — Hold at 37°C for 30mins – 32°C for 30mins
27°C for 20mins 22°C for 20mins

* Infected six mice with B9320 $V_6DC5_1V_1$ (10^{-1}) 32°C — Used ≈ 100µl of virus (titer ≈ 10^6 pfu ml^{-1}) intranasal (Anesthesized mice first). Also injected 2 of the same mice in the leg with some remaining virus.

Meanwhile harvested B9320 infected Vero's
32°C 10^{-1} undil } Now $V_6DC5_1V_2$ 4-25-94
37°C 10^{-1} undil }

Set up 14 mini preps to check the Xca/Hha construct in HB101. (Also) did 2 pBR322 minis for control (4.33kb)

To Page No._____

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

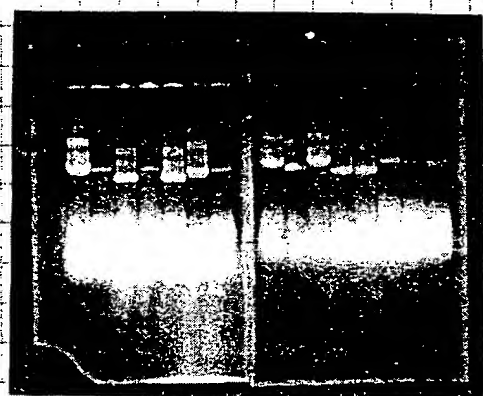
TITLE Mini prep analysis of HB101 Hhu/Xcu clones Project No. Book No. 73

From Page No.

Carried out mini prep DNA extractions on potential Hhu/Xcu clones in HB101

Prepare 2 x 1% Agarose gel for the 1st look analysis of these DNAs

* Ordered 25µg of pBR322 from Promega



Unfortunately it appears that none of the inserts are large enough to be the correct insert - will try inserting into pBR322 Bam HI site

NB IDEA - Clone the problem

Will proceed with two approaches

① Using Bam HI linkers, put the Hhu/Xcu fragment into pBR322 and will then transform a variety of hosts (HB101, SURE, ABLE C, K)

② Add RSV sequences downstream and upstream of the Hhu/Xcu insert in order to 'hide' the problem region

* Called Greg Duke about recombination def bacterial strain

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K. Clarke

Project No.
Book No.
TITLE Hha/Xcu RT/PCR

From Page No.

Called Stratagene about Yeast Cloning system — Options ① yeast with Centromere plasmids
② Non K-12 Strain of E Coli (ATCC
③ Use Baculovirus.

Carried out TCID$_{50}$s on B93:N V_6 DCS$_1$ V_2 37°/32°C
D.I./Undil
Will read next Mon/Tues.

Spun down 1ml x 2 of Nucleocapsid prep — One tube contained about 200μl of the 'old' nucleocapsid prep — Dried pellets — will set up 2 RT/PCR RXNS from each pellet.

Prepared fresh 1.85M 2-Mercaptoethanol

Ordered Xho → Bstu primer — will try to do 4.48kb RT/PCR RXN.

5' [redacted] 3'

Set up Kinasing of BamHI linker oligo (10μg) ~ 1.25hr at 37°C

10μl (10μg) of primer
5μl x10
5μl Spermidine 15mM
1μl ATP (10mM)
1μl Enz
H_2O to 50μl   1.25hrs at 37°C

Also set up 4 RT/PCR RXNS — Spun down ~1ml of EtOH ppt — dried resuspended in 4μl H_2O/4μl Hha → Xcu primer — Heated at 90°C for 2mins — Snap cooled added ~1μl of MethylMerc, 3mins RT, then ~1μl of β-MercaptoEtOH, 3mins RT then dispensed into RXN tubes and added 1μl of RT-ase → 1.25hrs at 42°C — Proceeded to set up PCR RXNs.

Set up O/N Xcu/Bam ④ and 3K sure

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by. | Date

Recorded by David K Clarke

TITLE Analysis of RT/PCR products / Preparation of Bam HI linkers. Project No. Book No.

From Page No.

Prepared 250ml broth for O/N plasmid prep"

prepared 1% agarose gel for analysis of O/N RT/PCR products

loaded 10µl of each RXN mixture.

Chloroform extracted RT/PCR RXNs and EtOH ppt^cd with a little glycogen

New New Old RNA Old RNA 1Kb ladder

★ Ordered New RT/PCR Kit (Perkin Elmer)

≈ 15ng/band in H/X RT-PCR products

Total of 300ng for 'New' and for 'Old'

Good - it looks as though the quality of the New RNA is also v good

Will Digest the 'Old' product with HhaI and ~~Bst~~ Bst1107I for subsequent ligation to the the 3.6K DNA and the Xca/Bam fragment

$\boxed{50\mu l}$ 2µl Enz

~1.5hrs at 37°C

Annealed 2µg of Kinased Bam HI linker ② to 2µg of Unkinased Bam HI linker ① In NTE

Heated briefly at 90°C

then put at 37°C for 30mins

Then " " 32°C for 30mins

Then " " 27°C for 20mins

then " " 22°C for 20mins

With 5min intervals between each annealing temp to allow cooling

Set up O/N 250ml broth cultures

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.______
Book No.______ **TITLE** Gel purification of Hha/Xca RT/PCR product

From Page No. __

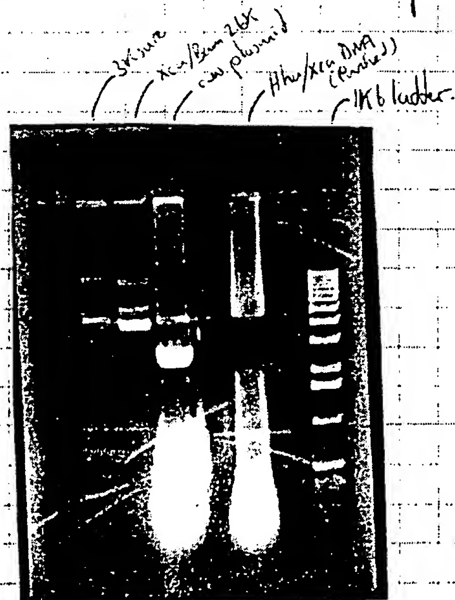
There is no HhaI enzyme in the freezer (or HinP1I – an isoschizomer of HhaI) – ordered 2,000 u of each

Spun down Bst1107I digested Hha/Xca RT/PCR DNA and stored dried at -20°C until ready for digestion with HinP1I.

Meanwhile run a gel of the remaining Hha/Xca RT/PCR product

Electroeluted the DNA and EtOH ppt^{ed} with glycogen.

Also carried out plasmid preparations of Xca/Bam (2.6K) and 3K sure using the Qiagen columns – Run out 5μl (of ~600) on an agarose gel.



Will digest the 3.6K clone with HhaI or ~~PstI~~ HinP1I and ~~the~~ KpnI and XhoI (to find out orientation in the MCS).

Will digest the Xca/Bam clone with Bst1107I and BamHI

It looks as though the 3K sure and Xca/Bam DNAs are okay and ≈ the correct size. Will know better following digestion.

3K SURE ≡ 100ng / 5μl^{-1} (Net 12μg)
Xca/Bam ≡ 1μg 5μl^{-1} (Net 12μg)

Can then reclone 3.6K/Hha-Xca/Xca-Bam frag back into pCR II vector → Sure → One shot etc.

To Page No._____

Witnessed & Understood by me.	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

TITLE Analysis of purified Hha/xca / Prep^n of pBR322 at VecFor.   Project No. Book No.   77

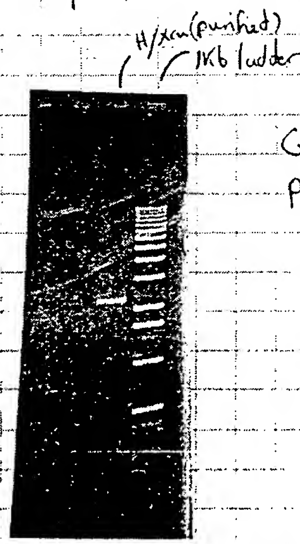
From Page No.

Set up BamHI digestion of pBR322 – which cuts in the Tet^R gene

50µl final vol / 2µl Enz / 2µg pBR322 / 2hrs 37°C /

Then Phenol chloroform extracted and EtOH ppt^ed prior to S.A.P (Shrimp Alkaline phosphatase treatment) 5' protruding ends

Also spun down gel purified Hha/Xca PCR product – resuspended in 20µl H_2O and analysed 2µl of this on a 1% Agarose gel



Good – there appears to be 50-60ng of purified Hha/Xa RT/PCR product in 2µl of the stock, giving a total of ≈ 500ng – This material will be used for the addition of BamHI linkers.

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by

Project No.______
Book No.______
TITLE Infection of Heps for more N capsid
S.A.P. of Bam cut pBR322 + gel purification
~~O/N ligation of Hha/Xca (pur) to Bam linker.~~

From Page No.__

Checked Hep-2 cells — they are ~ 70-80% confluent - will probably be okay to infect later this morning

Also did preliminary check on $TCID_{50}$ — again it seems that 32°C is the best temp for growth of RSV — this time undil gave a higher titer than dil. Will do a final Reading tomorrow.

10-30am Infected 10 x TC150s, each with 1ml of V_3H_1 32°C

Set up SAP RXN on pBR322 (2µg) in 50µl - with ~2µl of SAP 30mins at 37°C — added another 1µl SAP + 30min more incubation at 37°C — then added EDTA to 10mM and heat inactivated at 65°C for 15mins. Meanwhile prepared 1% Agarose gel for purification of SAP'd pBR322.

Will set up ligation of 4µl (100ng) of purified Hha/Xca DNA with BamHI linker, but first need to Kinase the Hha/Xca DNA - will Do all 500ng. Kinase reaction was as previously described in 50µl final vol; Due to recessed 5' end included a ½hr incubation following 70°C heat/snap cool (to denature the ends a little) to improve the efficiency of Kinasing; then phenol chloroform extracted and EtOH ppt^et - will set up the O/N ligation with linker tomorrow.

Meanwhile completed electroelution of pBR322 (Bam cut - phosphatased) and EtOH ppt^et following phenol chloroform extraction.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Greg W. Kibler		Recorded by David K Clarke	

TITLE Double Digestion of RV clones to purify Kinased Flanking sequences to Hha/Xca
O/N Ligation of Bam linkers to Hha/Xca DNA

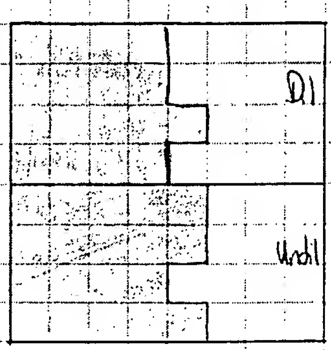
Project No.

Book No.

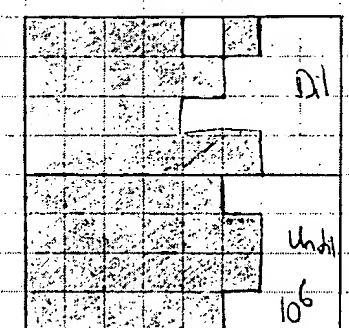
From Page No.

Checked c.p.e on infected Heps — also did a final read on the TCID 50s.

[redacted]



37°C
B9320
V_6DC5, V_2



32°C
B9320
V_6DC5, V_2

① Unpurified Bst 1107 I cut Hha/Xca fragment was digested with 1µl (10 u) of Hin P1 I in a 30µl vol at 37°C for ~2hrs

② ~5µg of clone 3.6K was digested with 4µl (40u) of Hin P1 I in 100µl at 37°C for ~2hrs

③ ~4µg of clone Xca/Bam was digested with Bst 1107 I (3µl - 24u) in 50µl for ~2hrs at 37°C

① needs to be gel purified on completion; ② and ③ should be phenol chloroform extracted and ppted with a little glycogen — then ② should be divided in two with one half digested with Xho I, and the other half digested with Kpn I to fix the orientation in the M.C.S. ③ should then be digested with Bam HI

prepared a 1% Agarose gel for analysis of purified pBR322 (Bam cut - phosphatased)
Will also run Bst/Hin P1 cut Hha/Xca RT PCR product

Will ligate 200ng of linker with 100ng of Hha/Xca fragment in 20µl O/N at 12°C
Spun down annealed linker and resuspended at [redacted] in H_2O on ice to prevent disassociation. Spun down Kinased RT/PCR Hha/Xca — gel purified and resuspended at [redacted] in H_2O

To Page No.

Witnessed & Understood by me. Date | Invented by | Date

Recorded by David K. Clarke

Project No.

Book No.

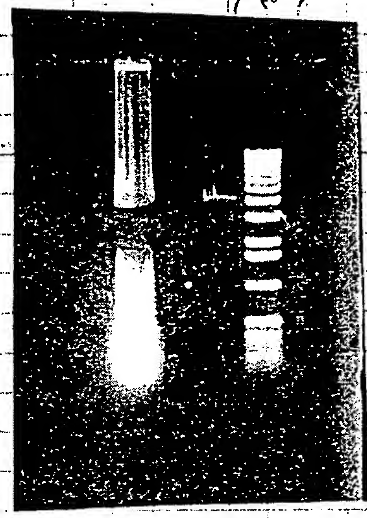
TITLE

From Page No. 79

Meanwhile carried out gel purification of HinP1 I / Bst1107 I cut $\frac{\text{Ith}}{\text{Xca}}$ RT/PCR

Eluted DNA from NA-45 paper - Phenol extracted and EtOH ppt^ed with 10µl of stock glycogen.

Purified pBR322 ladder



It seems that Promega have short changed us in pBR322 by a factor of 10. It appears there is only ~10ng of DNA in 1µl of purified, stock, phosphatased, and Bam HI cut. (Total ~200ng.) However I should have enough DNA for this experiment.

Also set up 2nd digestion on 3.6K, Xca/Bam, and in 20µl final vol to facilitate gel purificat^n

Split 3.6K DNA into 2 (4µg x2)

A) Xho I cut (with HinP1 I cut already done).

B) KpnI cut (with HinP1 I cut already done)

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K Clarke

TITLE Nucleocapsid Harvest / B9320 Infections / Digestion of Hha-Xca/linker with Bam HI

Project No.

Book No.

From Page No.

Infected Vero cells with B9320 D11 $V_6 DS_5 V_2$ } — undil. — 10^{-1} — 10^{-1} — undil.

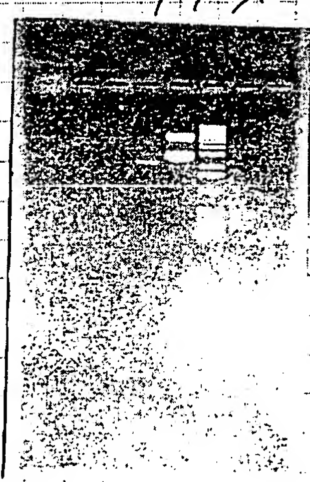
Undil

Absorbed ~ 3hrs at 37°C before feeding and putting at 32°C.

Began Nucleocapsid Harvest - as before - c.p.e looked very good on the 10 TC150s

Skipped second lysis buffer wash of pellet - went directly to T.E wash.

Phenol cloroform extracted ligated Bam linker / Hha-Xca purified RT/PCR DNA and etOH ppt^ed^ with glycogen: - will set up Bam HI digestion in 30µl (~2hrs at 37°C with 1µl (20u) of enzyme, tomorrow



The H/X frag has been trimmed with Hin PI I and Bst 1107 I and gel purified $\frac{1}{10}$th loaded on gel

It seems that the pBR322 stock is fine at 1µg µl^{-1}

The recovery of H/X frag is a little lower than expected (as for pBR322) should probably make fresh NA45 paper

Put on nucleocapsid spin for O/N Run - 29K 5°C 16hrs (2 gradients)

To Page No.

Witnessed & Understood by me, Serge W. Koble | Date | Invented by | Date

Recorded by David K Clarke

Project No.______

Book No.______

TITLE Genomic RNA harvest/Gel purification of 3.6K and X/B DNA

From Page No.__

Prepared some fresh NA45 paper for Electroelution of DNA from Agarose gels - stored at 4°C

Took off nucleocapsid spin - harvested nucleocapsid bands and collected by centrifugation after dilution in T.E buffer - Then resuspended in 500µl TE/pellet and set up SDS (0.2%) and proteinase K digestion.

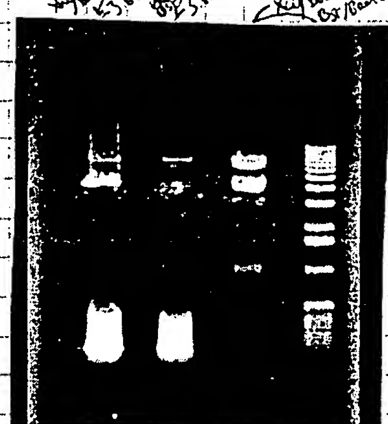
Set up Bam HI Digestion on linkered and gel purified Hha/Xca DNA (100ng) 2hrs at 37°C - 1µl Enzyme - Phenol chloroform extracted and EtOH ppt'ed with glycogen. Will ligate this DNA with Bam HI cut/phosphatased pBR322

Will use ~~100~~ 50 ng ($\frac{1}{4}$) of pBR322 prep and $\frac{1}{4}$ (~~50~~ 25 ng) of cut-linkered H/X fragment.

Will DO ~~100~~ 50 ng of pBR322 alone as con. Will set up ligations tomorrow

May need to recut linkered DNA due to high number of Bam HI sites generated by large amount of linker.

Kpn cut 3.6K Kpn cut 3.6K Xca/Bam clone Bx/Bam cut



Kpn cut is probably correct since it is the smaller DNA - will know better when the gel is rerun to check quantitation

Xca/Bam Digestion appears to have an Xho 1Kb frag! but it looks as though it is at sub-molar ratio - perhaps there is some 'Star' activity

Electroeluted all 3 DNAs - and phenol extracted/EtOH ppt'ed with glycogen.

To Page No.______

Witnessed & Understood by me, Sergei W. Kible | Date | Invented by | Date

Recorded by David K Clarke

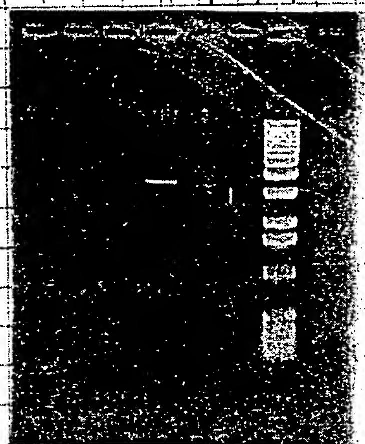
Interviewing of Potential RA's

TITLE B9320 Harvests/Quantitation of purified 3.6K and 2.6K clone DNA. Redigestion of Bam linkered Hha/Xcu DNA. Project No. ____ Book No. ____

From Page No. ___

Redigested Bam linkered H/X DNA with more Bam (to counter xess linker)
Used 3µl of Bam in 60µl for 3hr at 37°C — ∅ extracted and ppt'd with glycogen

3.6K Hha 3.6K Kpn 2.6K 1Kb ladder



The electroeluted products are in low abundance - about 50-100ng total in each - will need to check protocol to find out what is going wrong.
However - should have enough for 1 experiment.

Logically the slightly smaller 3.6K (KpnI) fragment is (HinPI I) the correct fragment

Will phosphatase both fragments tomorrow, and subsequently ligate with ~10-20ng of Hha/Xcu DNA, then ligate those products into KpnI/BamHI cut vector

Eco cut existing Vector - ligate - recut with Bam and KpnI - ligate in frag and transform
100ng insert : 50ng Vector

Harvested B9320 infections - now showing good c.p.e in all cases

10^{-1} V_6DC5, V_3 32°C < Dil / Undil
Undil " 32°C < Dil / Undil

refers to infecting virus (from previous harvest)

To Page No. ____

Witnessed & Understood by me, [signature] Date

Invented by Date

Recorded by [signature]

Project No.____
Book No.____

TITLE Eco Digestion of PCR II vector
Analysis of Bam cut, Bam linkered H/X DNA.
~~O/N ligation of linkered Bam cut H/X DNA to Bam cut phosphatased~~ pBR322.

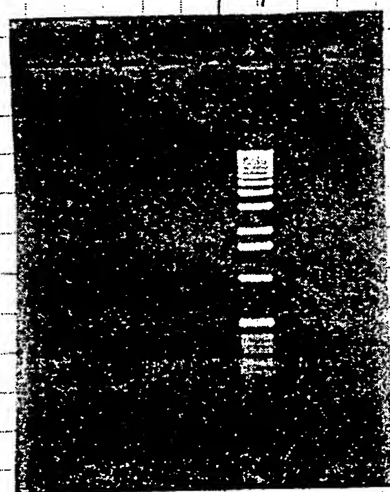
From Page No.__

Prepared L-Agar for tomorrows transformations — poured Amp[r] plates.

Prepared 1% Agarose gel for analysis of Bam Cut linkered H/X DNA — will analyse 1/4 of total (resuspended in 12µl H_2O.)

linkered BamHI cut

ladder



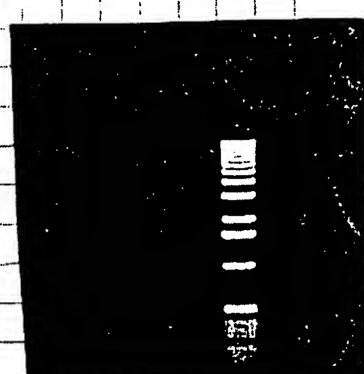
It may be necessary to Kinase the RT PCR primers to give a higher recovery of DNA (by eliminating this step from the procedure)

There is no obvious band of H/X (23K) DNA visible — decided to destain the gel a little to see if any band become more obvious.

(see gel below)

The red arrow below, points to the merest whisp of a band of DNA at the 23K position

Set up EcoRI digestion on PCR II T/A Vector — Ø extracted and EtOH ppt[ed]



Went ahead with O/N ligation -
Used 2µl Vector (≈ 20-30ng)
All remaining H/X linkered DNA (9µl)
heated at 65° for 3min to denature annealed linkers.
1.5µl x10 buffer
1µl Enz and H_2O to 15µl
left at R Temp O/N, along with an equivalent pBR322 only control

To Page No.____

Witnessed & Understood by me, Serge W. Kobl[illegible]

Date

Invented by

Date

Recorded by David K Clarke

000324

Bst4/Xho RT/PCR
Religation of EcoCut PCRII.
TITLE: Phosphatasing 3.6 KpnI and 2.6K DNA fragments
Transformations with pBR322+ linkered H/X DNA.

Project No.
Book No.

From Page No.

Set up phosphatasing rxns on 3.6K (KpnI) DNA and 2.6K DNA. (The low recovery level is probably due to insolubility of DNA after elution from NA45 paper due to the presence of gel agar) - Did 2x (15mins 37°C / 15mins 56°C) each time with 1µl of phosphatase (SAP) - Heat inactivated at 65°C / 10mM EDTA for 15mins, Then phenol chloroform extracted and ppt^ed

Set up religation of EcoRI cut pCRII - 2hrs at 37°C in 20µl 1µl enz. Then Phenol extracted and ppt^ed in preparation for KpnI/Bam double digest. (will save 2µl for gel analysis following religation)

Spin down 1ml of each N.Cap RNA prep^n and proceeded to set up RT/PCR RXNs as before

Set up bacterial transformations - using 5µl/transformation of O/N ligations between pBR322 and Bam linkered H/X DNA.

Used 'SURE' cells.
'ABLE K' cells
'DH5α' cells - (thyd as Con. also)

plated out 500µl from each on a separate large petri dish.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Sige W. Kole		Recorded by David K Clarke.	

Project No.______
Book No.______ TITLE Analysis of RT/PCR Products

From Page No.__

Checked bacterial transformants - There were ~100 on the large control plate, indicating that the phosphatasing was not complete - However it was

Galloway Lab Strain Collection

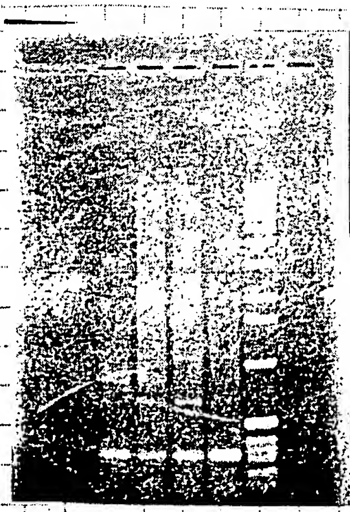
05/02/1994

Page 1

Location	232
Name	JC9387
Aka	S741 IN G. Smith's lab.
Total_size	0.000
Host_cell	Host cell
Vector	
Size_kb	0.000
Cut_with	
Insert	
Ins_size	0.000
Origin	
Release_by	
Resistance	
Comments	Used by Dan L. to maintain the HSV ori-bearing plasmids. AB1157 background[thr1,leu6,thi1,lacY1,galK2,ara14,xyl5,proA2,his4,argE3,str31,lsx33,mtl1,met-(weakly),su-]and recB21,recC22,sbcB15.
References	Construction of strain described in J.R.Gillen,Ph.D. thesis, UC Berkeley,1974. Lockshon and Galloway J.Virol. 58,513 (1986)

Set up BamHI digestion of religated EcoRI cut PCR II Vector - 2hrs at 37°C in 50µl with 1µl Enz

Set up Kinasing of H/X and X/H, used ~5µg of each in 50µl with 1µl Kinase ~1hr at 37°C



Gel looks discouraging - do not see the appropriate ~4.5Kb RT/PCR product expected.

Do not see Religated PCR II DNA either - however will proceed with Bam and KpnI digestion of this material

Set up O/N mini preps of JC9387 (from Galloway lab) in plain LB broth - no antibiotics.

To Page No.____

Witnessed & Understood by me, Surge W. Klob | Date | Invented by | Date

Recorded by David K. Clarke

TITLE: H/Xho and H/Xca RT/PCR RXNs.
KpnI digestion of PCRII (Bam Cut)

Project No.

Book No.

From Page No.

Set up KpnI digestion on Bam HI cut PCR II. — Phenol extracted and EtOH ppted.

Set up RT/PCR Hho/Xho (X1) and (H/Xca X4) — Used 3 x 1ml of EtOH ppt.
Used newest RT/PCR kit.
Used Kinased Oligo for Hho/Xca RT/PCR. Non Kinased for Hho/Xho.

Streaked out JC9387 bacteria on an L-B Agar plate; also froze down 2 x 1ml ampoules in 15% glycerol as long term stock.

Ordered some vector from Invitrogen pcDNA II

Prepared 0.1M CaCl2 (500ml) - will autoclave tomorrow

Will redo pBR322 cloning.

1. Use Kinased oligos for RT/PCR (as above)
2. Add Kinased linker and ligate (X100 x's)
3. Gel purify linkered DNA
4. Trim with Bam HI
5. Ligate into phosphatased pBR322.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Klein		Recorded by David K Clarke	

Project No._______

Book No._______

TITLE ~~Analysis of ligation components - 4 way~~ Analysis of RT/PCR products / TCID 50s

From Page No.__

Autoclaved 0.1 M $CaCl_2$ in preparation for Comp. cell production.

prepared 2 x 1% Agarose gels — one for analysis of RT/PCR products

one for analysis of the components going into the

Hha/Xho K' Hha/Xca

4 3 2 1

The Hha/Xca products are not as plentiful as I would have liked - will repeat with lml of EtOH ppt and Kinased Oligos again tomorrow.

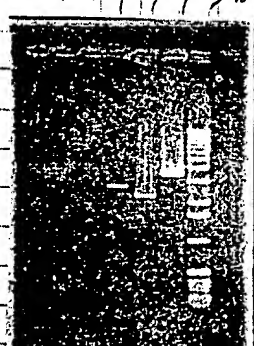
Meanwhile Chloroform extracted and EtOH ppt^ed with a little glyc

Will utilise only ① and ④

The Hha/Xho products look fine

Loaded 1% Agarose gel with 2.6K, 3.6K, Hha/Xca, along with Bam/KpnI cut ARI.

2.6K Hha/Xca 3.6K lad



Set up $TCID_{50s}$ on B9320

10^{-1} V_6 DCS, V_3 < 10^{-1} undil

Undil V_6 DCS, V_3 < 10^{-1} undil

Will use 1µl of 3.6K / 5µl of 2.6K / and 2µl of Hha/Xca and 150ng of pcII Vector.

To Page No.____

Witnessed & Understood by me, Date

Invented by Date

Recorded By

Gel purification of Hha/Xho frag.

TITLE: RT/PCR Rxn using Kinased Hha/Xca oligos. **Project No.** ______ **Book No.** ______

From Page No. ___

prepared 1% Agarose gel and run out Hha/Xho RT/PCR DNA ; for NA45 paper purification. Eluted DNA — phenol extracted and EtOH ppted

Set up RT/PCR Reaction (on 1ml of EtOH ppt ~~go~~ containing genomic RNA) using Kinased Hha/Xca primers.

Revised protocol for 3.6K, 2.6K, Hha/Xca and pcII plasmid cloning.

1. Digest Clones appropriately to give bands of DNA, phosphatase appropriately and then gel purify fragments
2. Double Digest pcII DNA - gel purify
3. Do tetrapartite ligation and transformation.

To Page No. ___

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kable		Recorded by David K. Clarke	

G. American
90 408 988-1776. Project No.______
Book No.______
~~Gp Sales X986 6853~~

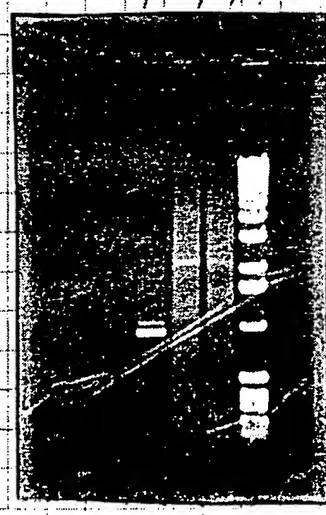
TITLE Gel analysis of RT/PCR products
Digestion of pcII DNA vector with Bam/KpnI.

From Page No.___

Prepared L-broth for growth of competent cells; prepared 400 ml L-Agar for Amp^+ plates —

Prepared 1% Agarose gel for analysis of Hha/xcu RT/PCR products and purified Hha/xho DNA.

Hha/xho
Hha/xcu V^r
Hha/xcu V^r
1Kb ladder



Interesting to see variation from Rxn to Rxn !!

Will gel purify all 4 (3) H/X K^+ DNAs tomorrow

set up BamHI digestion of pcII DNA – let run ~ 2hrs at 37°C in 50µl final vol, with 2µl stock enz.
Then phenol/chloroform extracted

Set up O/N ligation between PCR II Vector and Hha/xhoI DNA

Used 2µl Vector
1µl DNA
2µl x 2
14µl H_2O
1µl Enz
20

left O/N at 12°C

To Page No.___

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

~~Get purification of Hh/Xca K+ DNA~~
~~Ligation of Kinased linkers to Hh/xca DNA~~
TITLE ~~Get purification of Hh/Xca K+ DNA~~

Project No.

Book No.

From Page No. Transformation of 'SURE' and T/A cells with Hh/xho DNA
Kpn I digestion of Bam Cut pcII DNA

Prepared 1% Agarose gel for purification of K+ Hh/Xca RT/PCR products.

Meanwhile set up KpnI digestion of Bam Cut pcDNA II – in 50μl

37°C for ~2hrs 2μl Enz – Then froze away prior to gel

purification (tomorrow).

Set up 3 transformations Hh/xho in 'SURE' cells
Hh/xho in T/A cells
Xho/Xca in 'SURE' cells

Linker is at 100ngμl⁻¹ in H_2O ~ 10nt in length which is ~200 x smaller

than Hh/Xca(K+) – so [redacted] would require [redacted] of linker

Will shoot for a [redacted], but will wait until I get a sufficient quantity of pure Kinased Hh/xca DNA before proceeding – may need to adjust the RT/PCR conditions for the Kinased oligos i.e

① Raise the annealing temp
② Alter the rel. amount of oligo going into the RXN

will check DNA after linker ligation

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Sugr W. Kelle		Recorded by David K. Clarke	

92

Project No.
Book No.
TITLE Gel purification of Bam/KpnI digested pcII DNA.
Analysis of purified H/x K+ RT/PCR DNA.

From Page No.

Checked bacterial transformants — there are numerous clones on all plates.
However, ~~many~~ all of the clones on the T/A Hha/Xho plates had a slightly blue colour — streaked out the palest blue colour colonies on a master plate; also streaked out 'Hha/Xho SURE' colonies and 'Xho/Xca SURE' colonies, on a master plate — will set up O/N cultures tomorrow.

Prepared a 1% Agarose gel for gel purification of Bam/Kpn cut pcII DNA

X/H K+
1Kb ladder

Set up O/N J6 ~~3879~~ 9387

It looks as though there is ~10ng of DNA in 1µl = 100ng total remaining. Will ligate a (molar) 5X excess of Bam linker to this DNA O/N at 12°C (will check 1/10 on a gel)

This will be ~5ng of Linker (use 1µl of 1/20 dil^n of stock)

Must check M.p. of linker and ligate ~5°C below that (if consistent with a 12°C lig^n temp)

To Page No.

Witnessed & Understood by me. Date
Invented by Date
Recorded by David K Clarke

Preparation of Competent JC 9387 cells.

TITLE O/N ligation of Linker and H/X K+ RI/RR DNA

Project No.

Book No.

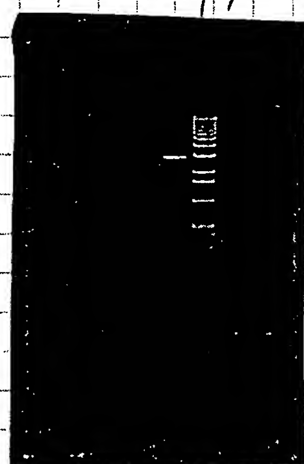
From Page No.

Prepared a 1% Agarose gel for analysis of gel purified Bam/Kpn (1μl from 11μl)

Inoculated 200ml L-b (prewarmed) with 1ml of O/N JC 9387

pcII DNA – BAM/KPN cut – Gel purified

1Kb ladder



Competent cell prep^n

NB All solutions and centrifuges etc should be chilled 0°–4°C

① Inoc prewarmed L-b (200ml) with 1ml of o/n culture

② Grow with vigorous shaking until $A_{600} \approx 0.3 - 0.5$

③ Chill on ice – then spin down at 3K r.p.m. for 3-4 mins.

④ Decant super and resuspend cells in 4/10 vol 0.1M $CaCl_2$ (chilled)

⑤ Let sit on ice for 30mins

⑥ Respin at 3K r.p.m. for 3-4mins

⑦ Decant super and resuspend at 4/100 orig vol in 0.1M $CaCl_2$

⑧ Leave on ice 1hr → O/N and/or freeze in EtOH/dry ice in 15% Glycerol

pcII DNA looks good at 2.9Kb

There appears to be ~ 50-100ng in 1μl – leaving 500-1000ng total remaining

Will set up 4 way ligation later in the week when I have competent JC 9387 tested for competence

To Page No. 94

Witnessed & Understood by me, Date Invented by Date

[signature]

Recorded by

David K. Clarke

Project No.______
Book No.______ TITLE ______

From Page No. 93

Checked $TCID_{50s}$ of B9320 – It is clear that at this point diluted virus does best during infection & either dilute a previous undil/infection stock to 10^{-1} before infecting or used a undil stock from a previously dilute infection – See final $TCID_{50}$ Read tomorrow

Set up O/N cultures of 14 clones each from
Sure Hha/Xho
Sure Xho/Xca
T/A Hha/Xho
+ 2 controls (T/A)

Set up o/n ligation between Kinased gel purified Hha/Xca DNA

Diluted stock primer 1/20th and used 1µl (5ng) in a ligation mixture containing ~ 100ng of Hha/Xca (10µl) in a final vol of 20µl.

2µl x10
1µl linker
10µl H/X.
6µl H_2O
1µl enz.

20µl O/N 12°C.

To Page No.______

Witnessed & Understood by me, Serge W. Kibbl[illegible] | Date | Invented by | Date

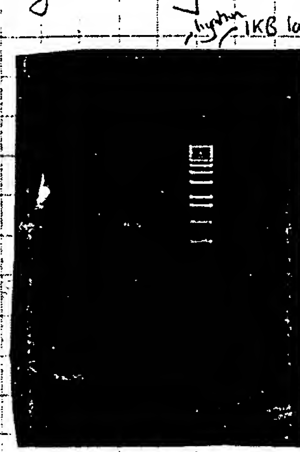
Recorded by David K Clarke

TITLE Trial Transformation of competent JC9387
Mini prep analysis of T/A and SURE H/Xho and Xho/Xca clones.

Project No. ______
Book No. ______

From Page No. __

Prepared 1% Agarose gel for analysis of O/N ligation products (linker + Kinased H/Xca

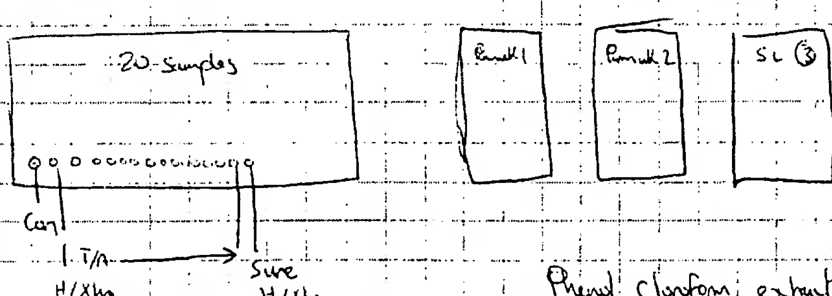


looks okay - there is still 5-10ng in this sample (1/10 of total).

Phenol choroform extracted and EtOH ppt^ed with 1μl of glycogen.

Went ahead with analys of 44 mini preps - 14 from each of the 3 transformation groups 'Sure Hha/Xho', 'Sure' Xho/Xca; T/A Hha/xho

Run out ccc DNA on x gels using pcII (2.9K) DNA as control - will be looking for ≈ a 2Kb difference for Hha/Xho and 2.3Kb diff. for Xho/Xca.



Phenol Chloroform extracted and EtOH ppt^ed Linkered K1 H/Xca DNA

To Page No. 96

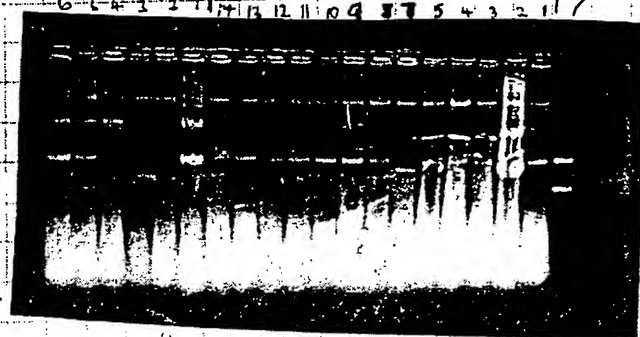
Witnessed & Understood by me.	Date	Invented by	Date
Guy W. Kibble		Recorded by David K Clarke	

Project No.
Book No.
TITLE

From Page No. 15

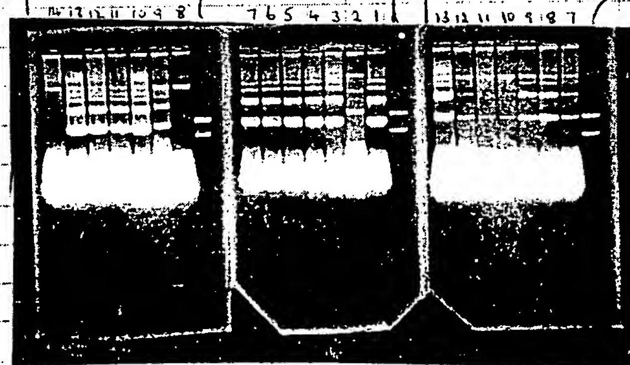
No 6 did not grow So was not run on gel

H/Xho in Sure
All are H/Xho clones in T/A vect
cut. pcII DNA



cut pcII DNA
cut. pcII DNA
(14) was not loaded due to lack of room.

Xho/Xca in Sure
H/Xho in Sure
cut pcII DNA



Clone No 2 in 'Sure' Xho/Xca looks as though it could be the one that I need

Most of the Hha/Xho clones look good in both 'T/A' and 'Sure' hosts

The Xho/Xca insert should be ~ 1,358, vs 976 for Hha/Xho
(This fits with the subtle difference in size of the ccc molecules).

Will digest 10µl of 2 with EcoRI + RNase A and 9 + 8 with XhoI and Hha
T/A H/Xho
Sure' H/Xho

2hrs 37°C

To Page No.

Witnessed & Understood by me,
Date
Invented by
Date

Recorded by

Restriction analysis of Potential Hha/Xho and Xho/Xca Clones. **Project No.**

TITLE BamHI digestion of linkered K⁺ Hha/Xca DNA **Book No.**

From Page No.

Checked transformation efficiency with pcII DNA in JC9387 - Looks very good (200pg gave v. many colonies)

22 colonies/pg ≡ 2.2×10^6/μg

Phenol Cloroform extracted (2) Xho/Xca in 'Sure'

(9) Hha/Xho in T/A

(8) Hha/Xho in 'Sure' and EtOH ppt'd prior to

double digestion (2) with Xho and Bst 1107 I

(9)+(8) Xho and Hha Did Xho digestions 1st.

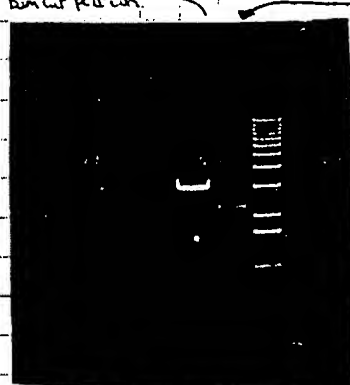
Also set up BamHI digestion on Linkered H/Xca DNA (K⁺) - with a Bam control (pcII DNA) Run 1% Agarose gel for analysis.

Will infect TC 75s with "10^{-1} V_6 DCS, V_3 - Undil" at 10^{-1} (1)

"Undil V_6 DCS, V_3 - 10^{-1}" Undil (2)

Used 0.75ml for each infection and adsorbed for ~3hrs.

Bam cut pcII con.



BamHI cut K⁺ linkered Hha/Xca DNA (1/5 of total) ~ 40ng remaining.

To Page No.

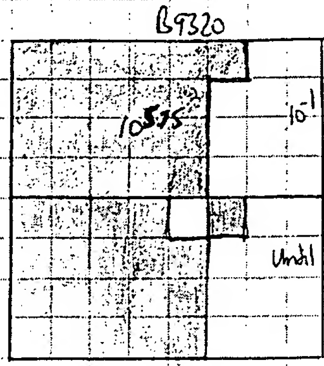
Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kebbe		Recorded by David K Clarke	

Project No.

Book No.

TITLE

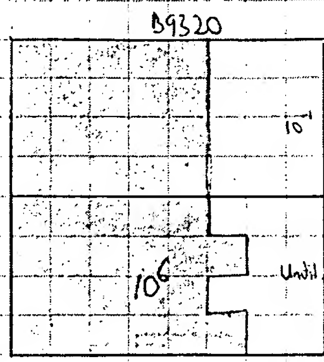
From Page No.



"Undil V_6 DCS, V_3"

Looks as though a general pattern is emerging – best to infect cells with dilute (10^{-1}) virus when coming from a 'high' titer stock produced from an undil infection

OR with undil virus from a 'high' titer stock produced from a dilute (10^{-1}) infection.



"10^{-1} V_6 DCS, V_3"

Completed double digestions on mini prep DNAs and froze away for gel analysis tomorrow.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

O/N ligation of 3.6, 2.6. H/xca int pcIIDNA and H/xca into pBR322.

TITLE Gel analysis of Potential Xho/xca and Hha/Xho clones

Project No.

Book No.

From Page No.

prepared L-Agar for tomorrows transformations ~400ml

poured agar plates - dried and stored at 4°C

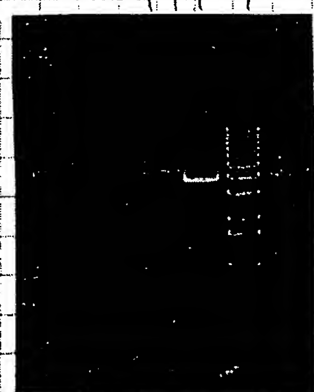
Prepared 1% Agarose gel and run analysis of (2) (8) (9) clones from double digestion

(9) Hha/Xho in TIA

(8) Hha/Xho insure

(2) Xho/xca insure

1Kb ladder



Good it looks as though all the clones contain the correct inserts, and the problem region(s) have now been cloned

Set up O/N ligation

(1) 4-way

Kpn1/HinP1 1µl 3.6K DNA (10-20ng)

Bam/Bst 5µl 2.6K DNA (10-20ng)

HinP1/Bst 2µl H/Xca DNA (5ng)

2µl pcII DNA (100ng)

2µl x10 buffer

7µl H2O

1µl Enz

12°C O/N

(2) H/xca to pBR322

2µl (10ng) H/xca - linkered K+ - Bam Cut

5µl (50ng) pBR322 - Bam Cut - phosphatased

10µl H2O

2µl x10 buffer

1µl Enz

20µl

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by

000339

Project No.______
Book No.______
TITLE Transformation of 'SURE', JC9387, DH5α with Hhu/Xca/Vector DNAs. — B9320 Harvest —

From Page No.__

Checked c.p.e on monolayers - not v. extensive - will leave harvest until later in the afternoon

Carried out the following bacterial transformations

5μl (of 20μl) from each ligation mixture for each transformation.

① SURE cells with pII DNA ligated to 3.6K, 2.6K, H/Xca DNA
② DH5α " " " " " " " "
③ JC9387 " " " " " " " "
④ SURE cells with pBR322 ligated to Bam linkered
⑤ DH5α " " " " " " "
⑥ JC9387 " " " " " " "

Plated out 50μl from each transformation mixture onto a large petri dish with Amp^{+} L-Agar

Harvested B9320 $V_6 DCS_1 V_4$ 10^{-1} and $V_6 DCS_1 V_4$ Undil — Carried out $TCID_{50}$s on both populations.

Interviewed Lisa Paganini.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature] Warwick K. Clarke	

Project No._______
Book No._______

TITLE: Muster plated potential Hha/Xca Clones

From Page No.__

Checked bacterial transformant - there are too many to count on the pcII DNA cells (Sure, DH5α, JC9387) - possibly due to incomplete cutting of one or other of KpnI or BamHI - Will repeat the transformation with 1μl of Ligation mixture and dilute out the transformed cells to /1/1000 / 1/100 / 1/10/

DH5α and SURE both gave colonies with pBR322 (JC didn't - probably due to lower transformation efficiency) - Plated out thirty for each set on a muster plate Amp^+ - will test on tet^+ plates tomorrow. —

To Page No.____

Witnessed & Understood by me, Sg W. Kihle | Date | Invented by | Date

Recorded by David K Clarke

000341

Project No.______
Book No.______ TITLE Testing tet$^{R/S}$ phenotype of potential Hha/Xa clones

From Page No.__

Prepared 200ml L-Agar Tet^{+} to streak out clones grown o/n on Amp^{+} master plates; if they contain an insert then the tet^{R} gene should be inactivated ≡ Tet^{S}

Continued to work on invention disclosure form.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kubler		Recorded by David K. Clarke	

Streaking out Muster plates with RSV clones.

TITLE: O/N minis of potential Hha/Xca clones. Project No. Book No. 103

From Page No.

Prepared 500ml L-Agar (Amp^r) for restreaking of clone banks - relevent clones only.

Will muster plate the following clones.

① 3K Clones (Vinu 5' end)

A) NCTA3
B) NCTA4
C) NCTA5
D) ~~NE~~ VNE14

Also 3.K in 'SURE'

② 1.5K Clone

A) Clone N° 2 only - it was the only one identified in batch of Wmini preps checked.

③ 2.25K Clone

A) 2
B) 4
C) ~~5~~ 6
D) ~~5~~ 7

④ 2.6K clone (also Known as Xca/Bam)

A) 2
B) 4
C) 8
D) 9

⑤ 2.3K (Hha/Xca)
To be fully figured out yet - So far it has been divided in two

Hha/Xho + Xho/Xca

A) 1
B) 10 } in T/A Vect

C) 8
D) 9 } in 'SURE'

A) ② in 'SURE'

⑥ 3.6K (Vinu 3' end)

A) 1
B) 3
C) 4
D) 7

Grew ON at 37°C

To Page No.

Witnessed & Understood by me, Date

Serge W. Kibble

Invented by | Date

Recorded by David K Clarke.

Project No.

Book No.

TITLE

From Page No. 103

Set up O/N mini-preps on potential DH5α pBR322 Hha/Xca clones

(Those which were showing Tet^S Amp^R)

To Page No.

Witnessed & Understood by me, Date

Invented by Date

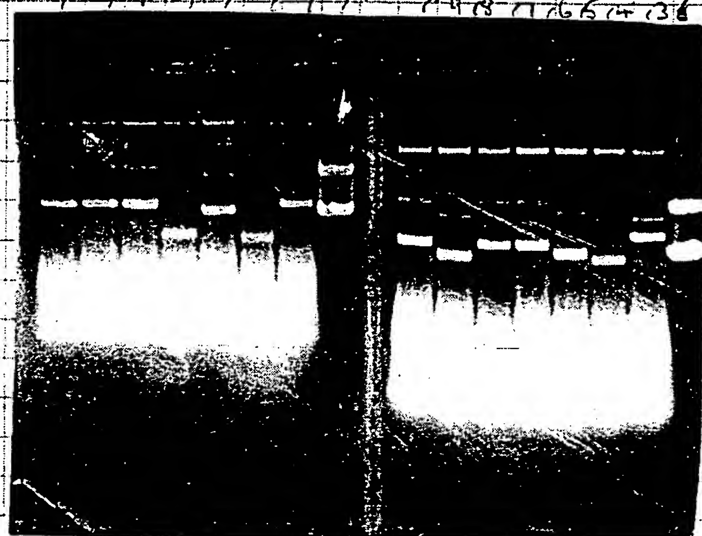
Recorded by David K. Clarke

TITLE Mini prep analysis of potential Hhu/Xca clones in pBR322 **Project No.**______ **Book No.**______

From Page No.__

Carried out mini prep analysis on potential Hhu/Xca clones

16 15 14 13 12 11 10 ... 9 8 7 6 5 4 3 pBR322



Stored clone banks at 4°C (Restreaked some of them)

It looks as though they have undergone various deletions and rearrangements

Possible non-deleted form of clone

Set up o/n mini preps of Clone ⑧ Hhu/Xho in Sure
⑦ Xho/Xca in Sure

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kell[illegible]		Recorded by David K Clarke.	

Project No.______ O/N Xho/Xca and Hha/Xho large scale preps.

Book No.______ TITLE Method for Hha/Xho and Xho/Xca link up.

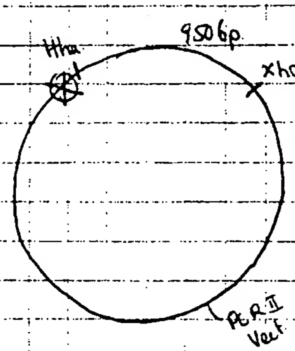
From Page No.__

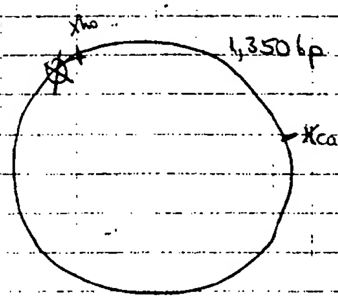
Put o/n cultures of Xho/Xca and Hha/Xho on ice prior to setting up o/n large cultures later this evening

A) Clone 2 Xho/Xca

B) Clones 7, 8, 9, 10 Hha/Xho

restreaked on new master plates.





cut ⊗ ~~Hha + Hinp I and~~ Xho cut

↓

Purify fragment.

~~May~~ need to determine orientation first.

Xho cut and gel purify ⊗ (Need to determine rel position of Xho I site

Checked $TCID_{50}$ – Will make a final reading tomorrow

Set up O/N cultures of (2) SURE Xho/Xca and (8) SURE Hha/Xho

To Page No.____

Witnessed & Understood by me, Serge W. Kuhlik | Date | Invented by | Date

Recorded by David K Clarke

Plasmid preps Hha/xho and Xho/Xca.

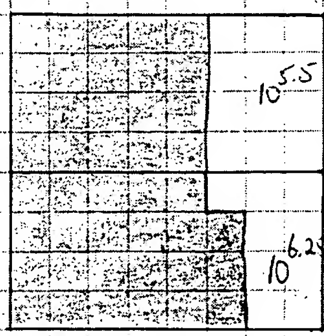
Project No.

TITLE Transformation with 4-Way ligation mixture **Book No.**

From Page No.

Prepared 400ml L-Agar Amp^{+} for transformation later today

Checked O/N growth of plasmid preps – Hha/xho was grown to a high cell density → Ice; let Xho/Xca grow for an additional hour or so as the cell density was a little lower; then proceeded with ~~mini~~ plasmid preps



B9320

$10^{5.5}$ V_6DC5, V_4 32°C

10^{-1}

$10^{6.25}$ B9320

V_6DC5, V_4 32°C

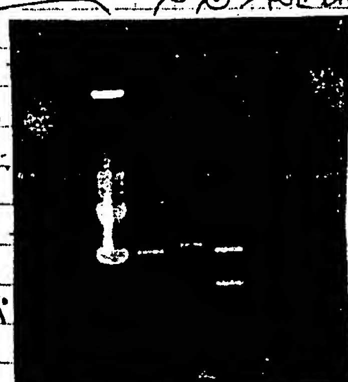
UNDIL

Will infect 6 x TC150 with Undil virus from the $10^{5.5}$ stock

and 6 x TC150 with 10^{-1} dil of $10^{6.25}$ stock

Set up bacterial transformation using DH5α, SURE, and JC9387 cells; Used 1μl of ligation mixture in each case – plated out 10^{-1}, 10^{-2} and 10^{-3} (200μl of each) from each cell transformation mixture.

⑧ ② pUC DNA



Possible Chromosomal DNA

actually looks as though the putative chrom. DNA is actually plasmid DNA.

GOOD; both plasmids Xho/Xca ② and Hha/xho ⑧ appear to have been stably amplified

[redacted]

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kimball		Recorded by David K. Clarke	

0000347

Project No.________
Book No.________

TITLE Master plate of Potential 8.5K Clones. Restriction analysis of 2 and 8

From Page No.__

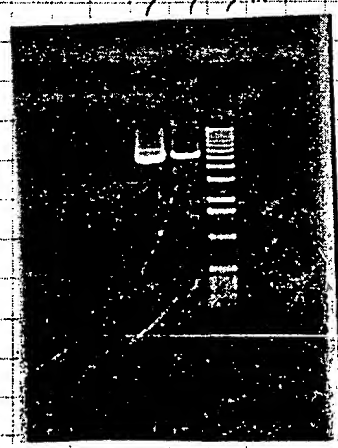
Checked bacterial transformations - streaked out 36 potential 8.5K Clones from SURE, JC9387, DH5α transformations

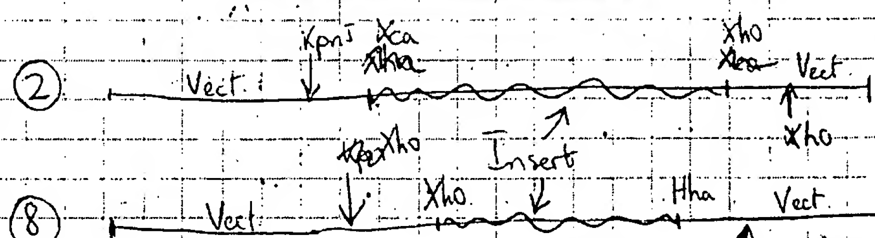
Will digest 20μl from each plasmid prep with both KpnI and XhoI - this will determine the orientation for each of the clones 2 and 8.

Phenol cloroform ~~ppt~~ extracted and EtOH ppt'ed between digestions

Prepared and run a 1% Agarose gel for analysis

8 2 1Kb ladder





Deduced from mini prep data

Need to Recheck 8 orientation - Cut with Xho alone - will give either one or two Bnds

To Page No.____

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

From Page No.___

Took potential 8.5K clones from incubator and stored at 4°C

Set up the following digestions

② - Xho I only (to check orientation of clone in vector - will give either one or two bands.

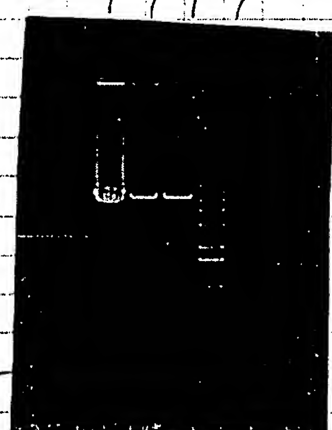
⑧ Xho I only (to check orientation)

② Bst 1107 I (to verify presence of this site)

Spun down 40µl ② and 20µl ⑧ - set up digestions

Prepared a 1% Agarose gel for analysis

xho 8 xho 2 Bst1107I ② 1K ladder



410bp fragment from MCS xho to CSV xho site.

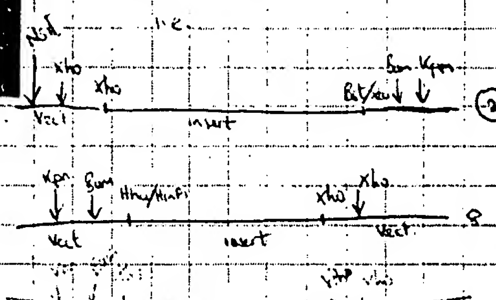
Will proceed by ~~Bam/Xho~~ EcoRI cutting ②

and ~~Bam~~/ Xho cutting ⑧

Everything looks fine

from gel can deduce that Xho I site of insert end and Xho I site of vector are on the same 'side'

i.e.



To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kohle		Recorded by David K Clarke	

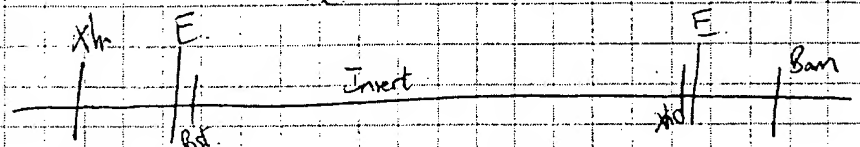
Project No.______
Book No.______
TITLE Infection of Veros for B9320 antigen.
Double digestion of (2) and (8) / O/N Minis for 8.5K clones

From Page No.__

Will spin down 200µl each of (2) and (8) for double digestion

Will have to flip the 'insert' of (2) around (within the EcoRI sites) to generate the correct configuration within the M.C.S; to do this will digest ~ 500ng of (2) then do religation and transformation (will check digests on a gel.

i.e



Will also ~~x~~cut (8) with Xho I (20µl)

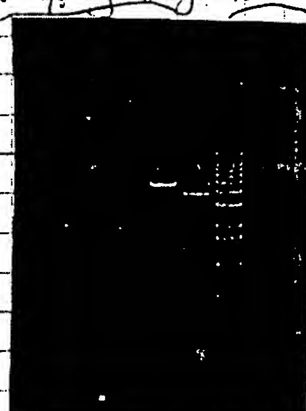
Infected 12 x TC 150s with B9320 virus

6 flasks with UNDIL V_6 DCS, V_4 32°C diluted 10^{-1} and 1.1ml used

6 flasks with 10^{-1} V_6 DCS, V_4 32°C Undiluted and 1.0ml used/flask

Adsorbed ~ 3hrs (to 3:45pm)

Meanwhile prepared a 1% Agarose gel for analysis of restriction digests



EcoRI (2)

Digestions look okay

Xho (8)

Set up O/N minis for potential 8.5K clones

To Page No.____

Witnessed & Understood by me, Date Invented by Date

Recorded by David K Clarke

Mini prep extraction and analysis for potential 8.5K clo **Project No.**

TITLE O/N ligation of EcoRI cut (2) **Book No.**

From Page No.

Carried out 30 mini prep DNA extractions on potential 'SURE' and JC clones which might contain the 8.5K clone

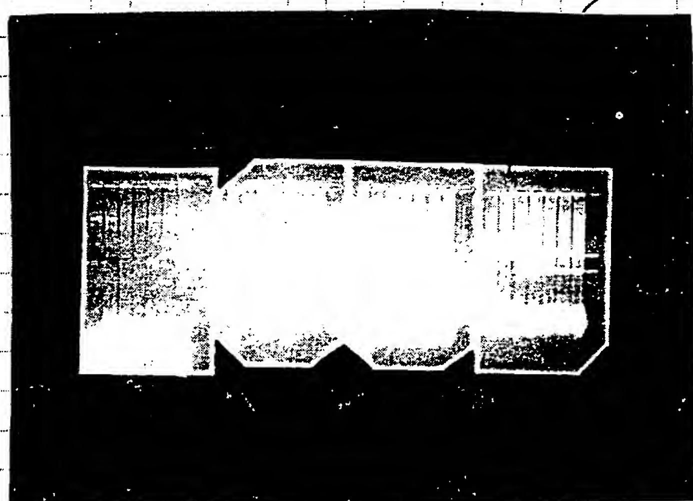
Prepared 4 x 1% Agarose gels and ran out samples with ccc pC II DNA as control marker

Only clone N°6 from the 'SURE' batch contained an insert, but it does not appear to be large enough to contain the

3.6
2.3
2.6
8.5Kb clone.

however - should see what size the insert is - it may be the 2.6K frag. (cut with ~~BstXI~~ NsiI)

Could try to do the same procedure in 3 steps using the Hind/Xho and Xho/Xcm fragments



Set up O/N ligation of Eco cut (2) (for religation) at R.Temp in 20µl with 1µl enzyme

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Gel purification of Xho cut (8)
Transformation of 'sure' cells with religated Eco Cut (2)
~~BstXI~~ NsiI digestion of SURE (6)-8.5K

From Page No.

Checked cpe on B9320 infected cells. Those infected with Undil virus are showing good cpe — those infected with Dil virus — not as advanced — Will harvest the 'Undil' virus today and possibly the dil virus tomorrow.

Set up ~~BstXI~~ NsiI digestion on SURE 6 (8.5K?)

2μl RNase A
2.6μl x10 buffer
20μl mini prep DNA

1μl ~~BstXI~~ NsiI

Prepared 2 x 1% Agarose gels - used one for gel purification of XhoI cut (8)

Used the other for analysis of NsiI cut Sure (6) (Bst 8.5K clone)

Prepared 200ml L-Agar and poured 6 Amp+ plates

Set up transformation of 'SURE' cells using 1μl of religated (EcoRI cut) (2) - to try and get the 2nd orientation, which should now give an insert band with XhoI digestion — Plated out 10^{-1}, 10^{-2}, 10^{-3}

SURE (6) cut with NsiI does not appear to contain the appropriate insert - it was most likely a dimer

NsiI cut SURE (6)' - NOT RELEVANT

ligation of Eco cut Sure (2) - it appears there is some unligated 'insert', but the upper band may be reformed ccc DNA. Will check ≥30 transformants to try and pick up both orientations

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

From Page No.__

Meanwhile carried out purification of B9320 Virus (Undil was showing good cpe) so proceeded with the 6 flasks infected with undil virus.

Clarified at 3K r.p.m for 10mins, then put on spin in SW 28 — 1.5hrs, 4°C 26K rpm — Decanted supers and resuspended each pellet in 200μl P.B.S. O/N on ice.

To Page No.____

Witnessed & Understood by me, Serge W. Kubble | Date | Invented by | Date

Recorded by David K Clarke

Project No.______
Book No.______

TITLE ~~O/N minipreps Master plates~~ Purification of B9320 / I.P. Boost of mice with RSV B9320

From Page No.__

Checked bacterial transformants – there are numerous colonies – will streak out 30-60 colonies on Amp^+, $X\text{-}gal^+$ agar

Went through B9320 purification of cells which had been infected with dilute virus (added $MgSO_4$ and Hepes buffer) – Did a freeze thaw cycle – did a card spin at 3K r.p.m for 10mins – then pelleted at 26K for 1.5hrs.

Set up mini cultures of the 2.25 and 1.5 clones – will inoculate them into larger (250ml) broths O/N and do plasmid preps tomorrow.

Prepared 1% agarose gel and analysed 1/10th of the total XhoI cut 8 Clone DNA

Gel purified XhoI cut 8

1KB ladder

Injected the previously IN RSV infected mice, each with 0.3ml. of a mixture (1:1) C.F.A : RSV B9320 in PBS (Virus from one T.C.150 was resuspended in 250µl PBS) I.P. One mouse died – perhaps from liver damage from the needle + internal bleeding

Total purified DNA = 500ng – 1µg. ≈ 25-50ng µl⁻¹

To Page No.____

Witnessed & Understood by me, Serge W Kable | Date | Invented by | Date

Recorded by David K Clarke

TITLE: Purification of ② Insert and Vector components after EcoRI digestion. Project No.

Book No.

From Page No.

Set up EcoRI digestions on

Prepared 1% Agarose gel for purification of all 3 DNA frags.

① 1μg pcII DNA

Prepared 1% Agarose gel for purification of bands

② 100μl (~2μg) of ②

Carried out plasmid purifications for 1.5K and 2.25K – 20min, 3K rpm.

Electro-eluted DNA from digestions onto NA45 paper (both digestions looked good)

Ran through plasmid purifications for RSV clones 1.5K and 2.25K

Eluted from Qiagen columns and precipitated.

Resuspended BA320 pellets in PBS (2μl) following O/N soak at 4°C on ice.

Froze away at -80°C – May use for 'Tail shot' for mice as a final boost for monoclonal Ab production.

Took a look at the mice – they don't look v. healthy – but should live.

To Page No.

Witnessed & Understood by me, Serge W. Kuhle | Date | Invented by | Date

Recorded by David K Clarke

Project No.
Book No.

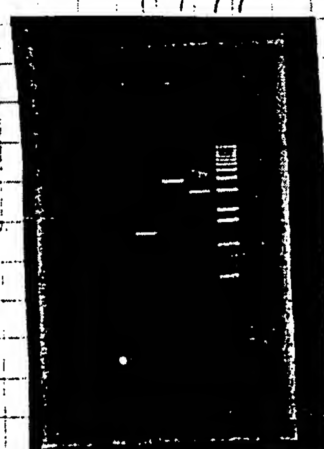
TITLE Gel analysis of purified fragments of clone (2). Restriction analysis of 1.5K and 2.25 K clones

From Page No.

Spun down gel purified Eco cut pcII DNA, and Eco cut (2) fragments to assess quality and quantity on a 1% agarose gel

Spun down 20µl each of 1.5K and 2.25K and set up EcoRI digest of 2.25K clone and Bam/Xho of 1.5K clone

Both XhoI and EcoRI have 5' protruding ends, so it should be fairly easy to phosphatase



Insert portion of (2) = 50ng/2µl

Vector portion of (2) = 100ng/2µl

pcII DNA = 50ng/2µl

Set up S.A.P. RxNs on the Vector portion of (2) and Eco cut pcII DNA, and XhoI cut (8) - Then phenol extracted and EtOH ppted

Prepared 1% Agarose gel for analysis of 1.5K and 2.25K digests

To Page No.

Witnessed & Understood by me, Date
Invented by Date
Recorded by

Project No.
TITLE Book No.

From Page No.

1.5K clone
2.25K Clone
1KB ladder

Good - both DNAs appear to be fine - correct size of insert etc.

2.25K digest was done on 20μl of EtOH ppt.

1.5K digest was done on 20μl of EtOH ppt.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Sze W. Kuble		Recorded by David K Clarke	

Project No.

Book No.

TITLE Ligation of Eco ② insert into pcII/Eco and Eco② vector portion

From Page No.

Ligated 100ng of pcII – Eco cut, gel purified and phosphatased with 50ng of insert from Eco cut ② (gel purified).

Also ligated 100ng of Vector from Eco cut of ② (also gel purified and phosphatased) with 50ng of insert.

Set up a Vector only (from Eco cut ②) ligation as a control to test the efficacy of the phosphatasing rxn.

left ligations at R.Temp O/N.

Prepared 9 Amp^+ Agar plates for tomorrows transformations.

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by

TITLE: Transformation of 'SURE' cells with O/N ligations from page (118). Project No.

Book No.

From Page No.

Set up transformations

Used 'SURE' cells – added 5µl from each ligation mixture to 100µl of comp. cells + 1.7µl β-mercap. – incubated on ice for ~ 1hr, then heat shocked at 42°C for 30 secs – incubated at 37°C ~1hr after addition of 900µl of SOC medium and then plated out 50, 100 and 200µl aliquots from each transformation mixture (included X-gal on the agar plates) – Put plates at 37°C o/n

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K Clarke

Project No.________
Book No.________ TITLE O/N Master plates of potential Xho/Xcu clones in either orientation.

From Page No.__

Checked transformants - control shows that there is a low background of religated vector - ligations into pcII vector appear to have been most efficient - as it generated the greatest N° of white colonies - hopefully some of these contain Xho/Xcu in the appropriate orientations. Will grow Master plates O/N.

Checked sequence for insertion of mutations (as markers for recognition of rescued virus). Selected region of the genome for mutation insertion is the coding and non-coding regions at the end of the 'F' gene.

Paper summary!

Set up o/n cultures of other Hhu/Xho clones to look for appropriate orientation.

Pme I — changed nucleotides

NON-CODING REGION 5' CCT AAT CAT GTT CTT ACA ATG [redacted] C AT CTG CTC ATA GAC
AAC CCA TCT GTC ATT GGA TTT

GTTT AAAC Pme I

Stu I — Changed nucleotides

CODING REGION 5' TTG TTA TCA TTA ATT GCT [redacted] G CTC TTA TAC TGT
L L S L I A V G L L L Y C
AAG GCC AGA AGC ACA CCA
K A R S T P

AGG CCT Stu I

Will order primers as soon as Hhu/Xcu clone is confirmed.

To Page No.____

Witnessed & Understood by me, Date Invented by Date

[signature]

Recorded by [signature: David K Clarke]

Mini preps of three remaining Hha/Xho clones

TITLE: O/N minis of potential re-oriented Xho/Xca clones

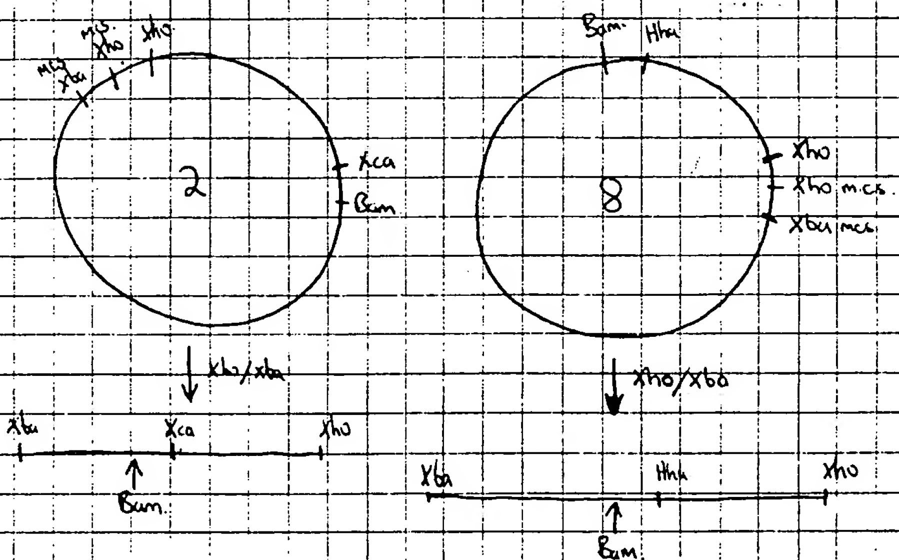
Project No.

Book No.

From Page No.

Carried out mini preps on two more Hha/Xho clones to check if they are in the same orientation as 8 — set up Xho I digestion on both as a test for orientation.

Also set up Xho/Xba I double digests on 2 and 8 for duplex approach to forming complete 2.3K clone



Can also try flipping 8 around (within the MCS - using the BstXI sites on either side of the clone). Will cut, do (fill in) (Blunt end) Xca and then purify Blunt ended insert, prior to religation ~~Phosphatase~~ and Vector portion needs to be phosphatased

To Page No.

Witnessed & Understood by me, Date Invented by Date

Serge W. Kirkle

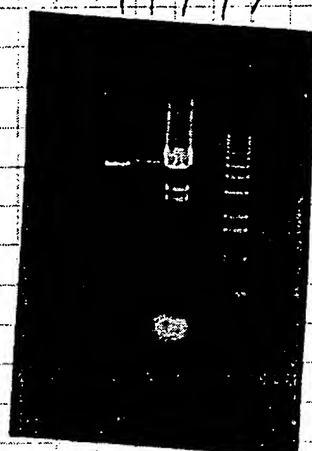
Recorded by David K Clarke

Project No.________
Book No.________ TITLE ________________

From Page No. 121

prepared a 1% Agarose gel for the analysis of the Xho/Xba I double digests and the Xho I digests of the Hha/Xho clones.

⑧ Hha/Xho.
⑦ Xho/Xca.
Hha/Xho ⑩ in T/A
Hha/Xho ⑨ in Sure
1KB ladder



Looks as though T/A clone is unstable after O/N growth.

9 SURE - may contain the correct orientation. (not quite enough DNA

Will try growing: 7, 9, 10, 11, 12, 13 as O/N cultures on Wed > Thur

Set up O/N minis of potential "Flipped" Xho/Xca clones in 'SURE'.

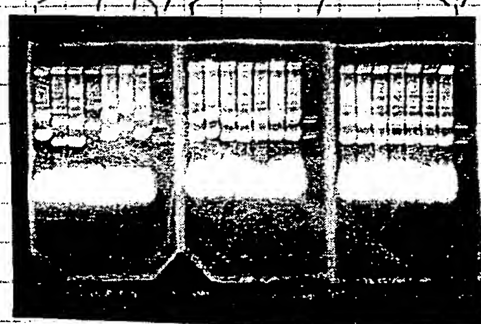
To Page No._____

Witnessed & Understood by me,	Date	Invented by	Date
Signature		Recorded by David K Clarke	

Project No.
TITLE Mini preps to check for "flipped" Xho/Xca
Book No.

From Page No.

Carried out 21 mini preps to try and identify possible 'flipped' Xho/Xca clones in either the original vector (2) or pcII plasmid. Did 14 samples from pcII transformation, and 7 samples from (2) transformation.



Clones (2) and (4) look like possibles - will phenol extract them and then EtOH ppt. prior to Xho I digestion.

Set up O/N mini preps of Xho/Xho clones 7, 9, 10, 11, 12, 13 in an attempt to find the 'other' orientation of DNA in the M.C.S.

Stored Xho I digestion of (2) and (4) above at -20°C O/N - will check on a gel tomorrow.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

Project No.

Book No.

TITLE Gel analysis of possible 'opposite' orientations of Xho/Xca clone

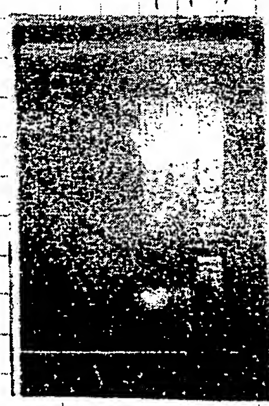
From Page No.

Checked O/N Xho/Xho clones – they have not grown well – will replate onto a master plate and grow O/N before redoing mini preps.

Prepared a 1% agarose gel and ran out Xho digested (2)/(4) (from yesterday)

Also restreaked remaining Xho/Xca (potential) clones – excluding those already tested.

Set up O/N mini preps for remaining 'Flipped' Xho/Xca clones (8–14)



Unfortunately neither (2) nor (4) are in the correct orientation, although (4) does look as though it contains the correct sized insert

It may be that the 'G' open reading frame will not permit cloning in the opposite orientation.

Will check the remaining clones to try and find the other orientation.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kemble		Recorded by David K Clarke	

Gel purification of Xho/Xba digested

TITLE further gel analysis of possible Xho/Xca clones (Flipped) Project No.

Book No.

From Page No.

Carried out mini-prep DNA extractions on another (remaining) 7 'Flipped' clones (8-14) – Ran out on a 1% agarose gel

Also prepared a 'deep' 1% agarose gel for purification of Xho/Xba digested ② (Xho/Xca) and ⑧ (Hha/Xho) clones

11 10 λcII marker

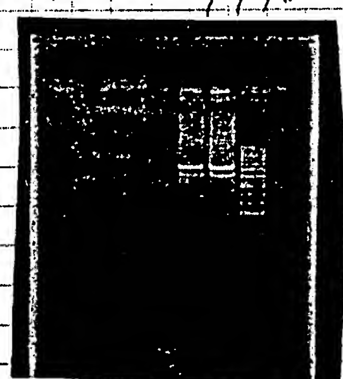
Proceeded with electro elution and purification of Xba-Xho cut ② and ⑧ ppt'd with a little glycogen

'Flipped' Xho/Xca clones 10 and 11, look as though they contain the correct sized insert but what is their orientation?

Proceeded to phenol cloroform extract and EtOH ppt

Then set up Xho I digestion to see if a 1.3Kb insert could be cut out, indicating the correct orientation

11 10 1KB ladder



Unfortunately neither 10 nor 11 are in the correct orientation

(Double bands are due to blurring during the photograph – camera is not working properly)

To Page No.

Witnessed & Understood by me, Seg W. Kuhle

Date

Invented by

Date

Recorded by David K Clarke

000365

Project No.
Book No.
TITLE Analysis of gel purified 2, 8 Xba/Xho cut
Phosphatasing of 8

From Page No.

Carried out gel analysis of gel purified Xba/Xho cut 2 and 8
Run 2µl of 20 for each

8 2 1Kb ladder

Also set up BamHI digestion of ~~Bsc~~ Xca/Bam clone to determine orientation of insert; likewise did a BstUI digest of the 3.6K clone, and will follow up with a Xho I digest tomorrow.

2 = 25-50ng Total 200-400ng

8 = 50-100ng Total 400-900ng

Proceeded to phosphatase all of 8 DNA - Then phenol chloroformed and EtOH ppt^ed

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kemble		Recorded by David K Clarke	

000366

Project No.
TITLE: Book No.

From Page No.

Span down BstUI cut 3.6K and set up XhoI digestion ~2hrs

Set up O/N mini preps for Hha/Xho clones 7, 9, 10, 11, 12, 13 to see if there were any in the opposite orientation to 8.

Also set up remaining Xho/Xca mini preps from original cloning.

Set up O/N ligation of 2 + 8 - phosphatased

Also did 2 and 8 on their own to check 'background'

Used 4µl of each ie 2µl x10
4µl 2 - of 20µl total ~80ng
4µl 8 - of 20µl total ~160ng
9µl H_2O
1µl Enz
20µl O/N R.Temp

To Page No.

Witnessed & Understood by me, Date Invented by Date

Serge W. Kubik

Recorded by David K Clarke

Project No.______
Book No.______ TITLE Transformations with ligated 2/8 Xho/Xba cut. Mini preps of potential Hha/xho and Xho/Xca clones

From Page No.__

Checked mini-preps - carried out DNA extractions on those which had grown.

Meanwhile prepared L-Agar for transformations - Did transformations, each with 5μl of O/N ligation mixture 2, 8, 2+8

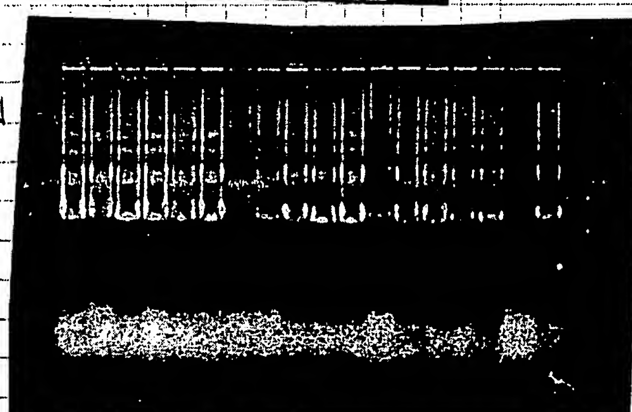
Plated out - 200μl /plate

Hha/xho clones

12 9 7 FC II DNA con

7, 9, 12 look as though they still contain the correct sized insert - in the others the ~~insert~~ plasmids have rearranged compared to their first analysis - following the transformation

Will phenol extract and EtOH ppt the remaining DNA for digestion and analysis tomorrow



None of these potential Xho/Xca clones appear to contain the appropriate insert

To Page No.____

Witnessed & Understood by me, Serge W. Kemble | Date | Invented by | Date

Recorded by David K Clarke

TITLE Gel analysis of digested 3.6K, 2.7K (Bam/Xca) Master plates for transformants.

Project No.

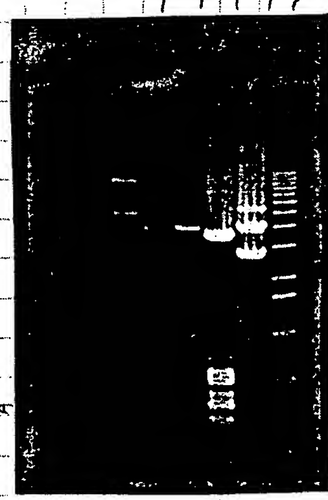
Book No.

From Page No.

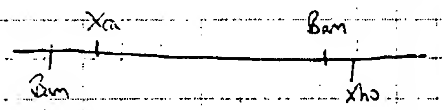
Checked o/N transformants - strangely many of the colonies are blue (in the control (2) - but not phosphatased (8)) indicating that transforming plasmid is deleted in the insert region to allow expression of β galactosidase. Will pick some of the white colonies and grow as O/N master plates.

Meanwhile set up XhoI/RsaI digests on H/Xho clones 7, 9, 12 from yesterdays mini preps to see if any are in the desired orientation.

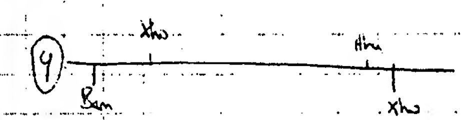
Prepared 1% agarose gel for analysis of digests.



It appears that the Xca/Bam clone (2.7K) is oriented as follows.



Both (9) and (7) appear to be in the opposite orientation to (8) (which is what I need) - Will grow ~~these~~ (9) up as a larger scale plasmid prep.



~~It also appears that 3.6K clone~~ It is not possible to identify the orientation of the 3.6K clone because BstUI had too many sites in the Vector.

Set up O/N minis of (7) and (9) for larger scale plasmid preps.

To Page No.

Witnessed & Understood by me. Date

Invented by Date

Recorded by

Project No.
Book No.
TITLE Xca/Bam double digests of xho/xca and 2.7K clones. Digestion of 3.6K clone to determine orientation of insert.

From Page No.

Checked o/n master plates - the vast majority of clones are expressing β-galactosidase and are blue - however there are a few whites which I will check by Mini prep.

Checked O/N Minis of 7 and 9 - they both have grown - stored on ice until ready to set up O/N large cultures for plasmid prep.

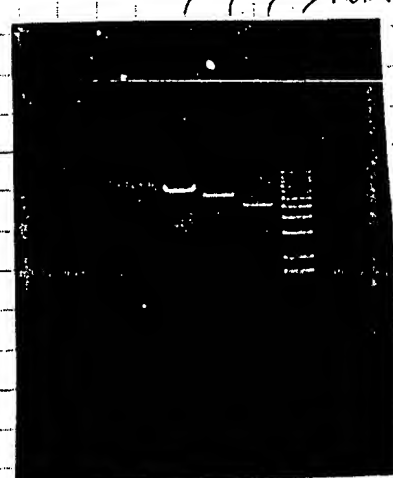
Will set up XcaI (Isoschiz. Bst 1107I) digestion on 1.3K Xho/Xca clone,
XcaI " digestion on 2.7K Xca/Bam clone

Will do digestions in 50µl and save 1µl for gel analysis.
Use 2-3µl Bst 1107I for ~2-3hrs

David S. helped (did) ~~so~~ the bleeding of the mice - retro orbital - stored the blood O/N at 4°C to allow clotting before spinning down to collect the serum

Prepared a fresh stock of 50mg ml⁻¹ Amp - filter sterilised and aliquotted → -20°

Set up O/N plasmid prep cultures for ~~Xhot~~ Xho/xho I. 7 and 9



Good it looks as though all the cuts went as expected.

Completed Bam HI cuts on the 1.3K, and 2.7K clones - froze at -20°C til tomorrow.
Phenol chloroform extracted Avr II cut 3.6K clone - EtOH ppt until tomorrow - will then cut with Bam HI

To Page No.

Witnessed & Understood by me. Date
Invented by Date
Recorded by David K Clarke

000370

Plasmid Preps for Hhu/Xho (7) (9) inv orientation to (8)

TITLE Bam Digestion of 3.6K clone + gel analysis. O/N Mini preps of potential Hhu/Xcu Duplex clones

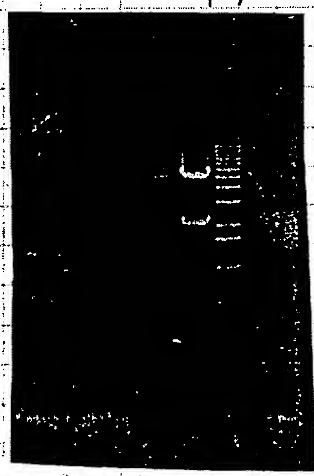
Project No.

Book No.

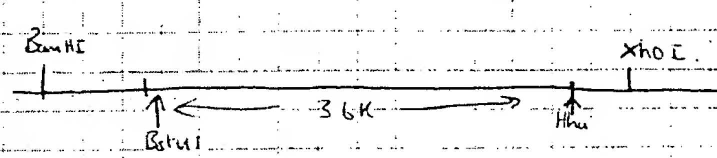
From Page No.

Set up Bam HI digestion of 3.6K clone (AvrII cut) - Ran it out on a 1% agarose gel

AvrII/BamHI cut 3.6K clone
1KB ladder



3.6K orientation in Vector



Will Hhu/XhoI cut (9) (next plasmid prep) and purify the frag (~1Kb) - can then ligate into Hhu/XhoI cut 3.6K (gel purified)

Spun down the clotted Mouse Blood for 5mins in the 'eppendorf' - then collected as much serum as possible from each - aliquotted and froze at -20°C - proceeded to let the blood clot further before purifying again for more serum.

Ran through plasmid preparations for Hhu/Xho clones (7) and (9) - stored as an etOH ppt at -20°C

Set up 20 O/N mini preps of potential Hhu/Xcu clones - included Kanamycin + Amp in half of them, just Amp in the other half.

To Page No.

Witnessed & Understood by me. Sean W. Kelk | Date | Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE Mini preps of potential 'duplex' Hha/Xca clones.

From Page No.

Went through mini preps on potential Hha/Xca clones - stored until sufficient time to do digestion analysis (Did phenol chloroform extraction and stored at EtOH ppt stage)

Will cut with Bam HI/ENbase A to check if correct sized insert is there.

Respun the mouse blood to collect remaining serum - froze serum with '1st round' stuff at -20°C

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by David K. Clarke

Gel Purification of Xca/Bam Cut 1.3K and 2.7K DNA FRAGMENTS. Digestion (BamHI) of potential Hha/Xca clones

Project No.

TITLE:

Book No.

From Page No.

Set up Bam HI digests on 1/2 Mini prep material of potential Hha/Xca 'duplex' clones - if the experiment was a success then Bam should give the appropriate 2.3K insert

Also prepared a 1% agarose gel and ran out Bam/Xca cut (2) 1.3K Xho/Xca clone and Bam/Xca cut 2.7K in order to purify the appropriate DNA bands.

The purifications went according to plan, and the bands were as expected.

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by David K Clarke

Project No.
Book No.

TITLE Analysis of BamHI cut potential Hhx/Xcu clones
Analysis of gel purified Xcu/Bam cut 1.3K, and 2.7K clones
Analysis of purified plasmids 7 and 9 Hhx/Xho

From Page No.

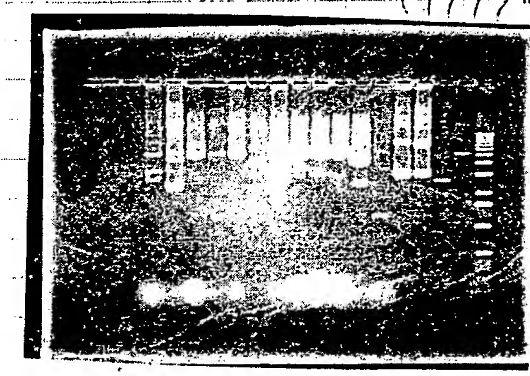
Prepared a large 20 well agarose gel (1%)

Ran out Bam HI digested mini preps of potential duplex Hhx/Xcu clones

Also looked at 2μl (1/10) of purified 1.3K clone DNA and 2.7K clone DNA

Also checked 1μl of 400μl of purified plasmid DNA for 7 and 9 Hhx/Xho
(Private Specification (8))

9 Hhx/Xho
7 Hhx/Xho
2.7K Insert
1.3K Vector
1KB ladder



100-200ng of 1.3K Vector

200-300ng of 2.7K insert

Both 7 and 9 look good
DNA conc. in both is ≈ 1μg μl⁻¹.

None of the Bam HI cut potential duplex clones are showing any promise of containing the Hhx/Xcu clone.

To Page No.

Witnessed & Understood by me.	Date	Invented by	Date
Gary W. Kibbe		Recorded by David K Clarke	

TITLE Phosphataseing of purified 1.3K DNA. Project No.______ Book No.______

From Page No.__

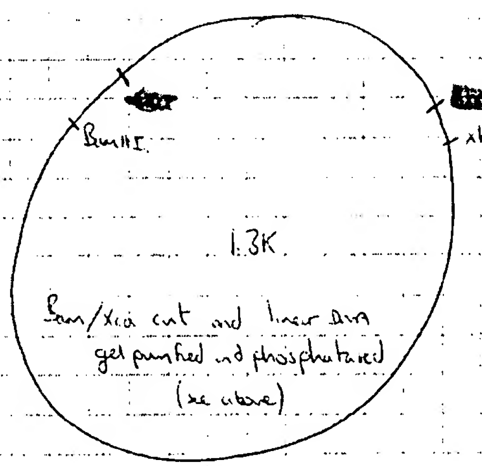
Prepared 400ml L-agar to restreak clone banks

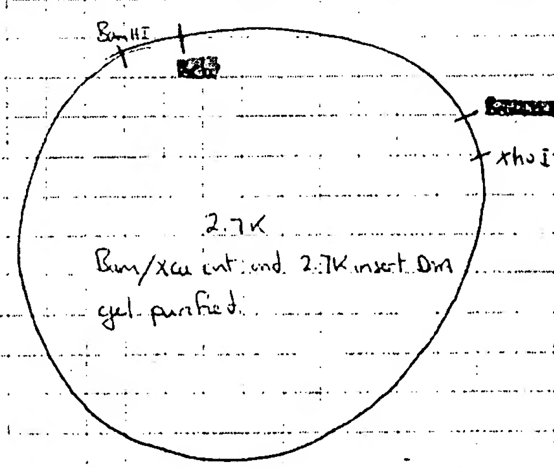
set up phosphatasing reaction on gel purified 1.3K DNA (vector) - 1hr at 37°C in 30µl - added a fresh ½µl of enzyme after the 1st ½hr of incubation

8µl DNA
3µl x10
18µl H_2O
1µl Enz
30µl.

Then phenol chloroform extracted - added 1µl of glycogen

Streaked out entire clone bank - incubated o/n at 37°C

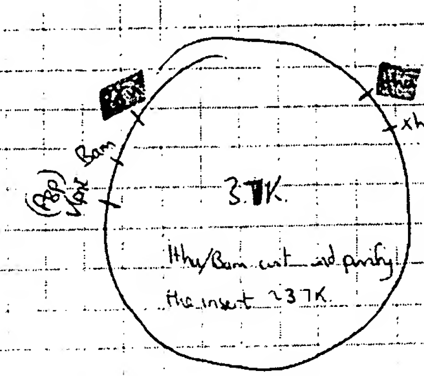


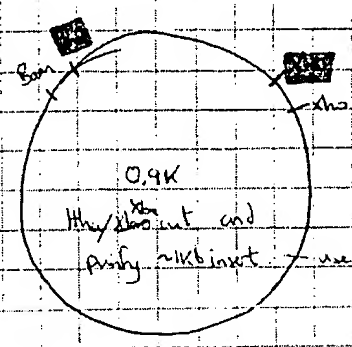


To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature: David K Clarke]	

From Page No. 135





– use fresh NA45 paper

Unfortunately there are many Hha I sites (31) in the vector !!

↓ Phosphatase thoroughly

Then titrate in increasing amounts of unphosphatased 3.7K DNA insert to find optimum ratio for a given amount of 3.7K added – purify the appropriate 4.7K DNA species? – trim with Xho I and ligate into Xho/Bam cut vector

(May want to electroelute into a dialysis bag for larger amounts of DNA)

Should cut ~ 10μg of 0.9K ≡ 2μg of insert
~ 5μg of 3.7K ≡ 2μg insert

Incubate 3.7K insert with a x5, x10 and x100 molar excess of phosphatased 0.9K insert – sample at 5mins – 30mins – 1hr, 2hrs at 37°C

Begin with a X10 molar Excess of 0.9K clone (phosphatased).

(need x4 as much of 3.7K to give a molar equivalent to 0.9K DNA).

For 150ng of 0.9 I will need 2μg (3.7K) for a 1:1 ratio "or" 200ng for a "1:10 ratio"

To Page No. ______

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by [signature]

Analysis of gel purified + Phosphatased 1.3K Vector DNA. **Project No.**

TITLE O/N ligation of 1.3K Vector and 2.7K insert DNAs. **Book No.**

From Page No.

Prepared a 1% agarose gel for analysis of 1.3K DNA - gel purified and phosphatased - loaded 1/5th (2µl) of total DNA.

Stored clone banks at 4°C after O/N growth at 37°C

Restreaked JC9387 - the colonies were very old though - so it may not grow - may need to go back to a stab.

Will also try to join the 0.9K and 1.3K clones by the following mechanism.

① XhoI/~~[illegible]~~ cut ⑦ or ⑨ 0.9K clones - and gel purify the fragment.

② XhoI cut 1.3K and gel purify - then phosphatase

③ Ligate and transform - look for 2.3Kb insert with Xho/Xca digestion.

There is between 50 and 100ng of DNA in 2µl of phosphatased 1.3K vector. Will ligate 50ng of insert 2.7K to 1.3K vector (i.e. 3µl vector)

Ligation RXN:

3µl Vector (1.3K): 100ng
3µl Insert 2.7K 50ng
2µl x10
11µl H_2O
1µl Enz
20µl O/N at 12°C.

Prepared dialysis tubing for electroelution purposes

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Sioux W. Kale		Recorded by David K Clarke	

Project No.
Book No.
TITLE Transformation with 1.3K/2.7K ligation mixture
Digestion of 0.9K and 3.7K clones

From Page No.

Prepared 300ml L-Agar for plates to do transformation with 1.3K/2.7K ligat" mixtures

Set up Hin P1 digestions of ~10μg of Hin/Xho (8) and 3.7K clones in 100μl – used 8μl Enzyme – digested for 4hrs at 37°C
10am → 2pm.

NB The Xho/Xca clone would not grow at 37°C on an agar plate – so could not maintain it as a bacterial stock – similarly for one of the 3.1K clones
(However have DNA (plasmid) for both clones – so should be able to transform more sure cells)

Redesigned 3.7K/0.9K splicing experiment

(1) will continue with Xba I cut of 0.9K plasmid (after HinP1 cut)

(2) will recut 3.7K with ~~HindIII~~ KpnI and Xho I – use isoschizomer Asp 718 or Acc65 I (Behringer, NEB)

(3) will purify both fragments from (1) and (2) above by electroelution.

(4) will phosphatase both fragments, then trim ~~0.9K with Xho I and~~ 3.7K with Hin P1 (and re-purify if necessary)

(5) Ligate 3.7K and 0.9K DNAs which should ligate predominantly at the HinP1 site – purify if possible – then with Bam and Xho

(6). Reclone into Bam and Xho I cut and phosphatased vect.

To Page No.

Witnessed & Understood by me, Gary W. Klein
Date
Invented by
Recorded by David K Clarke
Date

O/N Minis of potential Xho/Bam 4K clones

TITLE Xho I Digestion of 7 Hin/Xho. Project No. Book No.

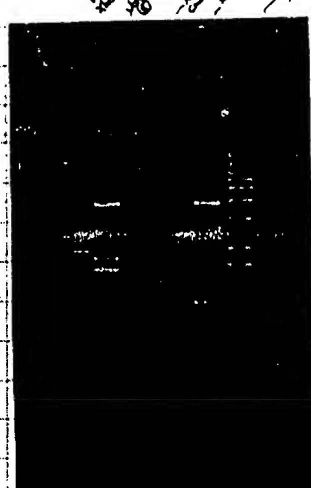
From Page No.

Checked bacterial transformants – there are many 'revertants' which are blue – indicating that many of the clones have undergone some type of rearrangement – there are also white clones which will pick and grow as mini preps.

Set up xho I digestions on 7 Hin/Xho and 1.3K clones in 50µl, 2µl enzyme, ~2hrs 37°C

Heat inactivated Mouse serum at 56°C for 30mins – Refroze at -20°C

Prepared a 1% Agarose gel and ran out 2µl (of 50µl) from Xho I digestions above, and from yesterdays digestion 3.7K Hind III/Xho I and Hin P.1/Xba I cut 0.9K clone.



Screwed up – there are 3 Hind III sites in the 3.7K clone – although it does confirm the size of the 3.5K clone

Set up 34 O/N mini's of potential Xho/Bam clones

Everything else looks okay although HinpI/Xba cut 8 has one partial product at ~1.2Kb. However the desired product is there at 0.9Kb.

Will recut 3.5K tomorrow (and purify Xho I cut 7 and 1.3 K fragments on SAT – use electroelution into dialysis tubing. Xho I/Asp718 or Acc65 I.

To Page No.

Witnessed & Understood by me. Date | Invented by | Date

Recorded by David K. Clarke

Project No.
Book No.

TITLE Gel purification of 1.3K clone and 0.9K frag of (7).
Digestion of 3.5K clone with Asp 718 and Xho I

TITLE

From Page No.

O/N Mini preps had not grown – the incubator had risen to 42°C – too hot
So set up two master plates of remaining 'white' colonies – will check later
this evening to see if they have grown sufficiently for 4°C storage, or continued
growth at R.Temp O/N.

Set up Asp 718 (Isoschizomer of KpnI) digestion of 3.7K clone in 40µl
using 2µl Enzyme for ~2hrs at 37°C – will then phenol extract - ppt, and
set up Xho I digestion – in 40µl with 2µl Enzyme ~ 2-3hrs.

Meanwhile prepared a 1% Agarose gel and ran out Xho I digested 1.3K Clone,
and 0.9K clones – cut out appropriate gel slices – eluted into
dialysis tubing and then collected and phenol chloroform extracted, and EtOH ppt^ed
with glycogen. – Will phosphatase the 1.3K DNA, and then analyse both
purified DNAs to quantitate prior to ligation and transformation.

Checked Master plates (see above); they have grown sufficiently to store at
4°C. Will set up Mini preps on Sun Evening

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by David K. Clarke	

Witnes

TITLE: Gel purification of Xho I/Asp 718 cut 3.6 DNA. Xba I/Hin p I I cut ⑧

Project No.____ Book No.____

From Page No.__

Prepared a 1% Agarose gel for purification of DNA bands - ran out fairly long to get good separation of DNA bands. Excised bands with a razor and eluted into dialysis tubing. Then phenol extracted and etOH ppted with a little glycogen. Will phosphatase and do gel analysis appropriately tomorrow.

As Expected the 3.6K DNA co-purified with the vector 3.9K DNA - Eluted both together. Will digest mixture with vector specific enzymes after phosphatasing and trimming with Hin P I I — then repurify 3.6K insert

use a lot!

The partial digestion of ⑧ on page 139 (XbaI/HinpI) is probably due to Dam/Dcm methylation of plasmid DNA - However ~ 60% of the DNA was cut - should probably trim with XhoI before cloning

e.g ① Phosphatase both ⑧ - 0.9K DNA and 3.7K DNA (EL Eluted)

② Cut 3.7K with HinpI and another enzyme with site(s) only in the Vector

③ Purify 3.7K DNA again.

④ Ligate 3.7K and 0.9K in appropriate ratios

⑤ Trim with XhoI and do ligations into (XhoI/Asp 718 cut Vector / XbaI/Asp718 " ")

To Page No.____

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K. Clarke

Project No.______
Book No.______ TITLE Alkaline Phosphatase treatment of Gel purified DNAs

From Page No.__

Set up SAP reactions on the following Electro-eluted DNAs

(1) XbaI/Asp718 cut 3.7K DNA
(2) XbaI/HinPI cut (8) – 0.9K DNA
(3) XhoI cut 11.3K DNA

all have 5' protruding ends, which facilitates phosphatasing.

Set up RXNs in 50µl final vol.

5µl x10 buff
44µl H_2O
1µl Enz
50µl

added an extra 1µl of enzyme after 30mins at 37°C and incubated for a further 30mins.

Then phenol chloroform extracted and EtOH ppt^ed.

Set up O/N mini preps of potential Xho/Bam clones X 34

Will run them alongside the 8.T 2.7K Xca/Bam clone to identify possibles

To Page No.____

Witnessed & Understood by me, Sean W. Kibble | Date | Invented by | Date

Recorded by David K Clarke

TITLE Mini prep analysis of potential xho/Bam Clones.
Neut. Tests on Mouse antisera.

Project No.

Book No.

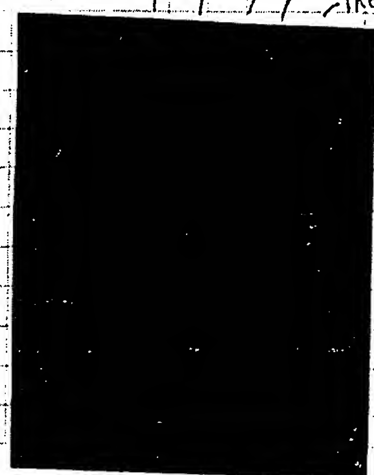
From Page No.

Carried out 34 mini preps to search for potential xho/Bam clones –

Will run gels later today

Prepared a 1% Agarose gel for analysis of Gel purified – phosphatased DNAs

3.7K pGP 718 xho I cut
1.3Kb xho cut
③ Hinc I / Xba cut
⑦ xho I cut
1Kb ladder



Quantitation.

① ⑦ = 100ng ≡ 2µg

② ⑧ = 50ng ≡ 1µg

③ 1.3K = 100ng ≡ 2µg

④ 3.7K = 50ng ≡ 1µg

Meanwhile Ran RSV neutralisation test

1) Diluted B9320 V_6 DC5 V_4 32°C 10^{-1} ($TCID_{50}$ $10^{5.5}$) to give 50 $TCID_{50}$ per 100µl in MEM containing 10% final G. Pig. Comp.

2) Made serial 2-fold dilutions of each mouse antiserum 1/10 to 1/160 (after diluting serum initially 1/5. Used 10% G pig serum in MEM for dilutions.

3) Added 100µl of diluted virus to each eppy containing A serum ... Then (Included a no a serum con.) incubated at 37°C for ~1.5hrs

To Page No.

Witnessed & Understood by me, Serge W. Kelle

Date

Invented by

Date

Recorded by David K. Clarke.

Project No.

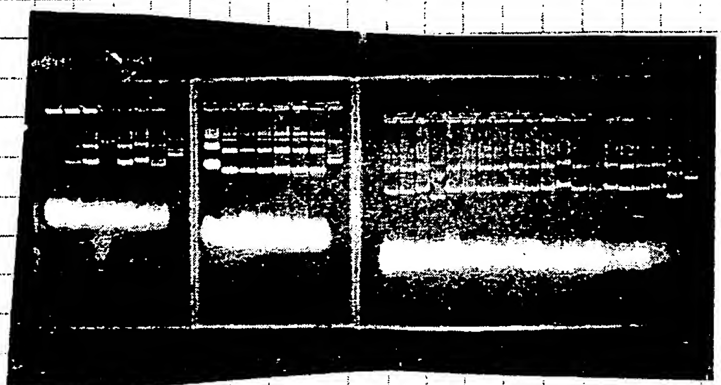
Book No.

TITLE

From Page No. 143

Meanwhile, carried out a $TCID_{50}$ on diluted virus to verify titer.

(see official protocol on p. 145)



Unfortunately none of the 34 mini preps show the correct sized insert.

Should try transforming JC-cells - call Galloway for Mini prep advice.

To Page No.

Witnessed & Understood by me.	Date	Invented by	Date
Sey W. Klle		Recorded by David K Clarke	

RSV NEUTRALIZATION ASSAY

1

MATERIALS

- VIRUS GROWTH MEDIUM
  500 ml Eagle's Minimal Essential Medium
  5 ml glutamine (200 mM)
  5 ml Pen-Strep (10k U + 10k µg/ml)
  5 ml fetal bovine serum

- VIRUS
  Thaw frozen amp of titered virus at 37^0. Immediately dilute in virus growth medium so that there are approximately <u>100 TCID</u>$_{50}$ virus particles per 100 µl. Guinea pig complement may be added (final concentration of 10%). ← MA Bioproducts Cat # 30-956J 5ml #28.35
  (800)-231-3352 Order ~ 30 ml

Dilute virus by ≥ 1/1000
1/10 1/10 1/10
into 800 µl MEM 100 µl G.Pig Comp.

- ANTIBODY
  The neutralization assay can reliably detect as little as 0.1 µg/ml of antibody. At antibody concentrations greater than 100 µg/ml, non-specific "neutralization" occurs. Therefore, you should dilute test purified antibodies to a concentration between 20µg/ml and 100 µg/ml. If you are testing unpurified polyclonal sera, make stock dilutions of 1/5 so that the final concentration of the first dilution tested is 1/10.

Do 1 dil without G.pig complement.

- CELL MONOLAYERS
  Assay is performed using TC96-well cell monolayers which are about 90% confluent and 2 to 3 days old.

- TC96 INCUBATION PLATES

PROTOCOL

Dil antibody G.pig complement? — does this contain G.pig complement too.

1st

1. Add 100 µl of virus growth medium to ~~wells~~ eppy tubes of incubation plate.

2. Add 100µl test antibody to first well, and make serial two-fold dilutions by transferring 100 µl across the plate. Test antibody dilutions should be set up in duplicate.

3. Add 100 µl of diluted virus to each well containing 100 µl of diluted antibody. Incubate virus-antibody mixture at 37^0 for 60 minutes.
   NOTE: Make sure that you save a little of the diluted virus — you need to do a TCID $_{50}$ quantitation of your diluted virus in order to confirm that you actually added about 100 particles per well. This is called the "back-titration", and is crucial for interpreting your neutralization assay results.

Do a "can't type incubation" with complement but no Ab. at 37°C and one at 37°C no comp.

4. At the end of the antibody-virus incubation period, flick the cell growth medium out of the TC96 cell monolayers, and replace with the antibody-virus mixture.

Need 1×96 well plate for Neut test.
1×96 well plate for controls.

5. After incubating for an appropriate period of time (~ 4 to 6 days for RSV), examine each each well for cytopathic effect.

6. Flick medium off, and stain with glutaraldehyde and crystal violet. Rinse with water. Endpoint is highest antibody dilution that provides protection from CPE.

Witnessed & Understood by me,	Date	Invented by	Date
George W. Klein		Recorded by David K Clarke	

Project No._______

Book No._______

TITLE HinpI/PvuI digestion of 3.7K DNA
EcoRI digestion of miniprep (31)
O/N ligation of 1.3K and 0.9K DNA

From Page No.__

14

Set up PVU I digestion on purified 3.7K DNA, to remove the Vector portion of the DNA — will repurify from an agarose gel

Phenol/chloroform extracted and EtOH ppt[ed] — then set up Hin P I digestion to trim one end — froze at -20°C — will repurify tomorrow from a 1% agarose gel

Also set up EcoRI digestion on mini prep (31) — to check the extent of inserts etc

Checked in with Stratagene on their Yeast Shuttle Vectors — they are sending out the literature

Set up O/N ligations of 1.3K XhoI cut Vector and 0.9K XhoI cut insert (Phosphatased vector)

Used 3µl Vector ~ 100ng
1µl insert ~ 100ng
2µl x10 buffer
1µl enzyme
H_2O to 20µl   O/N at R. Temp

To Page No._____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

TITLE: Transformation with ligated 0.9K/1.3K
Gel purification of HinpI trimmed 3.7K DNA.

Project No.______
Book No.______

From Page No.__

Prepared 300ml L-Agar for transformations - Amp$^+$.

Set up transformations - 5µl of each ligation mix added to 'SURE' cells

Also set up transformation with 1.3K clone - ccc DNA - (Dried down 1µl from EtOH ppt - resuspended in 5µl H_2O and used 1µl for transformation.)

Prepared a 1% agarose gel for gel purification of Trimmed HinpI/Asp 718 cut 3.7K DNA.

Called Denise Galloway for hints on mini-preps

It looks as though the PvuI/HinPI digestions did not go to completion because there is still some doublet left, indicating that not all the vector was digested

If repeat 3.7K purification will digest with Asp718/Xho and PvuII, then purify and phosphatase - then trim with HinPI and repurify - Will wait and see what is obtained with 0.9K/1.3K ligation.

To Page No.______

Witnessed & Understood by me, Serge W. Kolle | Date | Invented by | Date

Recorded by David K Clarke

Project No.______ O/N minis of potential Hha/Xca Clones

Book No.______ TITLE Gel analysis of purified HinPI trimmed 3.7K DNA.

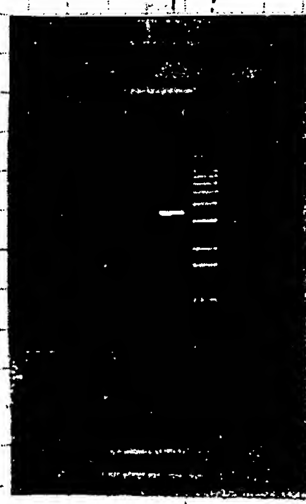
From Page No.__

Checked transformants — Virtually all the colonies produced by transforming with the 1.3K ccc plasmid are blue – indicating radical rearrangement of the 1.3K DNA insert. There may be a couple of white colonies which will be colony purified. There are some white colonies on the 0.9K + 1.3K plates — will pick them as O/N mini preps and onto master plates.

Run out 2µl (of 20µl) from re-gel purified 3.7K DNA HinpI trimmed.

HinPI Trimmed 3.5K DNA — Gel purified

1KB LADDER



Colony purified and/or picked the white colonies from the 1.3K ccc transformation.

Picked the white colonies from the 1.3K + 0.9K ligations and set up O/N minis + Masters.

There is 100-200ng of DNA of the correct size ~ 2µg of total DNA.

Can now proceed with ligations with 0.9K DNA.

Will use in µg equiv. for each ligation which will give a X3 molecular ratio of smaller 0.9K DNA

Need to ~~XbaI~~ XhoI cut (check on gel) and Asp718 cut pCDNA II. — Gel purify and phosphatase

Will start by annealing 100ng of each at 37°C for 1hr → Gel

(1µl 3.5K + 2µl 0.9K)

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by [signature] David K Clarke	

000388

Mini preps of potential Hha/Xca clones. Project No.

TITLE Checking Neutralisation Assays. Book No.

From Page No.

Went through mini prep procedures for sole potential 1.3K clone, from ccc transformation. Also potential Hha/Xca clones from 0.9K + 1.3K ligation

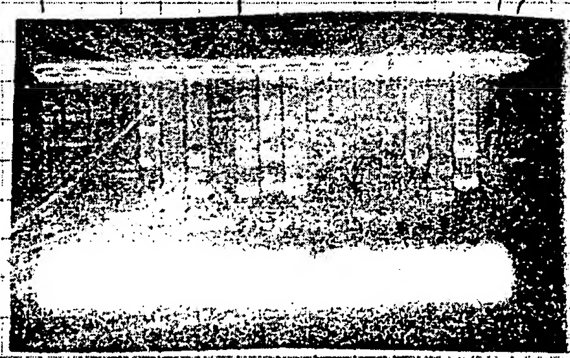
prepared 1% gel, and ran samples with Xho/Xca plasmid as marker.

Checked virus neutralisation assay - it is v difficult to interpret, since the complement seems to have adversely affected the cells (and virus?) - The complement may not have been suitable for tissue culture work; i.e. it may not be sterile, or may contain factors toxic to the vero cells. Will repeat the neutralisation using 3-10 times the amount of virus, and leave out the complement.

Lisa's

② - "Supercoiled" clone

ccc 1.3K plasmid



To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K Clarke

Project No.______

Book No.______ TITLE Back to work Preparation and planning for the rest of the week

From Page No.__

Back from San Diego today.

Phenol chloroform extracted potential Hhx/Xca clones and potential 1.3K clone (derived from straight transformation); EtOH ppted

1.3K clone = 2 super

Hhx/Xca = 6 7 8

Will be able to digest with BstX1 to determine the size of the insert DNA in these clones — should give two DNA bands; one at 1.7K and one at ~600bp

Wrote up Bi-monthly report on the RSV project

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
George W. Clark			

Recorded by David K Clarke

Digestion of Mini prep DNAs to check for 1.3K and

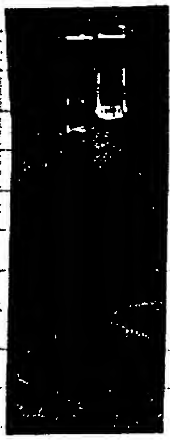
TITLE 2.3K inserts. Ligation of 3.7K and 0.9K DNA

Project No.

Book No.

From Page No.

Set up ligation between purified Rbp 718/Hha (HinpI) cut 3.5K DNA and purified 0.9K (Hha/Xba cut)

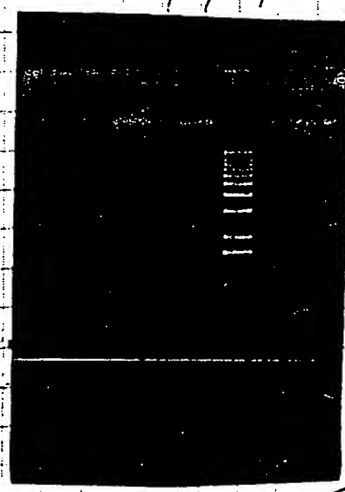


DNA cut so all is well

Used ~ 100ng of 3.5K and a X3 molar ratio of 0.9K (~100ng).

1µl of 3.5K DNA
2µl of 0.9K DNA
2µl x10
14µl H_2O
1µl ENZ
20µl

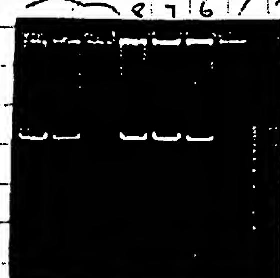
Meanwhile got Lisa to check that XbaI is indeed cutting (8) - as should be the case to give HinpI/Xba cut



Clearly all of the 3.5K and 0.9K DNAs are ligating to each other, giving high mol. wt. bands. There is also a little DNA of the ~4.5K size, which would be what we want.

Will double the amount of 0.9K DNA in the ligation and cut the time to 15mins at 37°C.

Also carried out EcoRI digestion of potential 1.3K Mini prep (2) DNA and BstXI digestion of potential Hha/Xba clones (6) (7) (8)



None of the potential Hha/Xba clones were good, however the 1.3K clone is good, so will make a glycerol stock from an o/n culture tomorrow.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
George W. Klebe		Recorded by David K. Clarke	

Project No. ______
Book No. ______

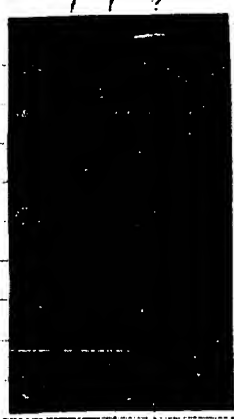
TITLE Ligation for 0.9K to 3.5K DNA – modified protocol
O/N Mini prep of (2) - 1.3K for glycerol stock.

From Page No. __

Tried setting up another ligation between 3.5K and 0.9K DNAs.

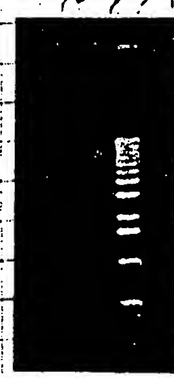
This time doubled the amount of 0.9K DNA and halved the incubation time.

1µl 3.5K DNA
4µl 0.9K DNA
2µl x10
12µl H_2O
1µl Enz
20µl — 15mins at 37°C – sampled ½ at ~~15~~ 7mins



Still not enough 0.9K DNA – will redo ligation with 8µl of 0.9K DNA

1µl 3.5K DNA
8µl 0.9K DNA
2µl x10
8µl H_2O
1µl Enz — 15mins at 37°C – sampled at 7mins
20µl



Obtained Zymolase from Ying (ICN supplier)

Will proceed by XbaI/Asp718 cutting pcII DNA (check after 1st cut) and gel purification.

Set up O/N 1.3K ((2) super) from remaining Mini prep culture (from last week)

To Page No. __

Witnessed & Understood by me, | Date | Invented by | Date

Serge W. Kuhn

Recorded by David K. Clarke

TITLE Long term storage of 1.3K Clone; Double digestion of pc II Vector (Xba/Asp 718)

Project No.

Book No.

From Page No.

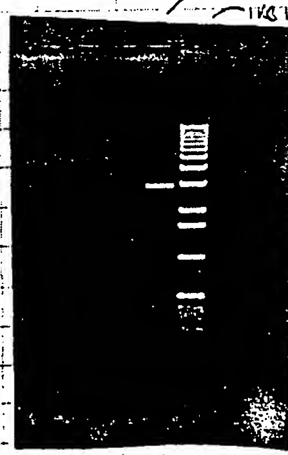
Checked on growth of 1.3K clone 'Super' 2 - it had grown so set up glycerol stocks (0.15ml glycerol + 0.85ml O/N culture) and froze away at -90°C.

Set up Xba I digestion of pc II DNA (~2μg) in 20μl final vol with 1.5μl of enzyme - Prepared 1% gel for analysis of 100ng ($\frac{1}{20}$th)

Streaked out 1.3K clone from O/N mini prep onto ~~agarose gel~~ agar Amp+, and grew O/N at 37°C

* Lisa carried out a mini prep extraction on 1.3K O/N culture + EcoRI digestion - to ensure that the O/N culture does indeed contain the correct insert. *

gel worked - it will have to be repeated.



There are trace amounts of uncut ccc and open circle plasmid, probably due to Dcm/Dam methylation of the XbaI site. However will go ahead with gel purified XbaI/Asp 718 cut.

phenol chloroform extracted XbaI cut pcII DNA - EtOH ppted and then set up Asp 718 digestion for ~ 3hrs in 20μl with 1.5μl Enzyme - Froze at -20°C prior to gel purification.

To Page No.

Witnessed & Understood by me, Serge W. Kl[illegible] | Date | Invented by | Date

Recorded by David K Clarke

Project No.______
Book No.______
TITLE Gel purification of Asp718/XbaI cut pcII DNA. Patent writing.

From Page No.__

Took o/n 1.3K clone from 37°C - it had not grown well - restreaked some of the colonies on a fresh Amp^+ plate and grew o/n at 37°C.

Prepared a 1% Agarose gel for gel purification of XbaI/Asp718 cut pcII DNA

Ran out DNA - excised DNA band and electroeluted - Phenol chloroform extracted and EtOH ppt^ed with some glycogen.

To Page No.____

Witnessed & Understood by me, George W. Kleb | Date | Invented by | Date

Recorded by David K Clarke.

Project No.

TITLE: Ligation of (0.9K+3.5K) into gel pure pcII DNA. Book No.

From Page No.

Patent writing!

Restocked the T.C Room!! Checked Temps + H_2O levels – Will check CO_2 levels tomorrow evening

Took 1.3K ② 'Super' from 37°C incubator – it appeared to have grown fairly well – stored at 4°C.

Set up the 0.9K/3.5K ligation – then added the gel purified pcII vector (200ng total added) in X1 lig buffer

Gave 10mins at 37°C

7µl 0.9K
1µl 3.5K
2µl X10
9µl H_2O.
1µl Enz.

20µl

O/N R Temp.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
George W. Kohl		Recorded by David K Clarke	

Project No.______ Transformation with 0.9K + 3.5K + PCII DNA.

 Book No.______ TITLE Neutralization Assays with mouse serum.

From Page No.__

prepared L-agar - amp$^+$ - 300ml to streak out transformants

Lisa prepared SCE solution (for Yeast cloning protocols)

Sorbitol, Sodium Citrate, EDTA } SCE. Also prepared 5M K^+ Acetate.

Carried out bacterial transformation with 0.9K + 3.5K ligated into pcII DNA

Used 5μl of O/N ligation mixture for transformation - plated out on Amp$^+$ Agar containing Xgal and IPTG - incubated O/N at 37°C

Also plated out 10^{-1} diluted transformed cells.

Set up Virus Neutralisation assay - diluted R9320 (V_6 DC5, V_4 32°C 10^{-1})

$10^{-1} \rightarrow 10^{-1}$ (5ml) ≈ 5×10^3 pfu ml^{-1}. Incubated 100μl with 100μl of each of the 5 mouse antisera - diluted serially two-fold (in 100μl final vol)

$\frac{1}{10} - \frac{1}{20} - \frac{1}{40} - \frac{1}{80} - \frac{1}{160}$.

~1hr at 37°C Also did back titration - to get a $TCID_{50}$ reading for the diluted virus used. Run a No antiserum control.

Lisa set up O/N 1.3K cultures; one from an agar plate one from a broth.

To Page No.____

Witnessed & Understood by me, [signature] Date

Invented by Date

Recorded by David K. Clarke.

TITLE O/N Minis + Master plates for potential 3.5K + 0.9K Clones in pcII

Project No.______

Book No.______

From Page No.__

Checked bacterial transformants - there are many blue colonies - but there does appear to be a few whites - will pick them later and set up master plates

Lisa proceeded with mini prep(s) - of 1.3K grown in broth/Agar (The broth derived culture had not grown v. well)

Called Stratagene for yeast stuff - Should be here by tomorrow.

Prepared 40% PEG (4000) solution, 10mM Tris (pH 7.5) - autoclaved

Checked in with Qiagen on their columns for use in plasmid purification from yeast. They suggested dissolving the pellet (after Isoprop ppt^n) in T.E then adding Q.B.T → Column

(Use small vol of T.E and then x10 vol of Q.B.T

Lisa's gel shows that the clone does indeed contain the 1.3K insert (After EcoRI digestion)

She will check the clone derived grown as a broth tomorrow

To Page No.____

Witnessed & Understood by me.	Date	Invented by	Date
[signature]		Recorded by David K Clarke	

Project No.______ O/N Yeast culture / Prepⁿ for Yeast Cloning.

Book No.______ TITLE Mini preps of potential 0.9K+3.5K in pcII Vector

From Page No.__

Checked Master plates at 37°C - many have turned blue - will not do those as mini-preps - stored O/N mini preps at 4°C until ready to do mini preps.

Received Yeast Cloning Vectors + Host strains from Stratagene - stored strains at -80°C, and Vector at -20°C

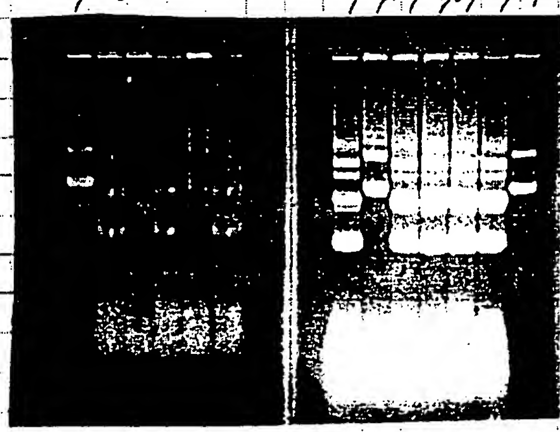
Prepared 1 Liter of 0.1M Lithium Acetate. - autoclaved

Ordered 2 x 96 well plates for Mon for neutralization assay (for Mon)

Carried out Mini preps of all 'white' clones from master plate - Run out on a 1% agarose gel with 3.6K clone as a marker (stored Master plate at 4°C)

prepared YPD Broth for growth of yeast cells

Set up O/N YPH 499 culture in YPD broth (~3ml) at 30°C



N° 11 looks like a good candidate.

A couple of Lisa's minis may also be okay

To Page No.____

Witnessed & Understood by me, Date | Invented by | Date

Recorded by David K. Clarke

Preparation of Competent Yeast Cells

TITLE Analysis of Mini prep DNA by digestion

Project No.

Book No.

From Page No.

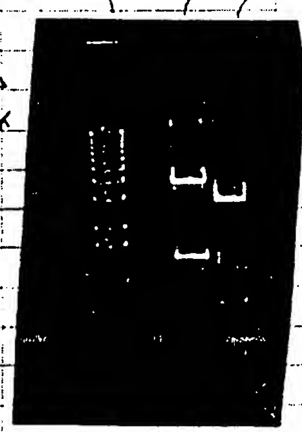
Checked growth of Yeast O/N culture - it hadn't grown yet - so left at 30°C for a further 24hrs. Meanwhile prepared some YPD Agar for plates in order to have a yeast stock on a plate.

Set up BstUI digestion of mini preps (11) + 2 of Lisas

Checked the Neutralisation assay - it looks good - most mouse antisera are showing good neutralisation activity, some out to 1/80 and 1/160 dilution - will make a final reading tomorrow.

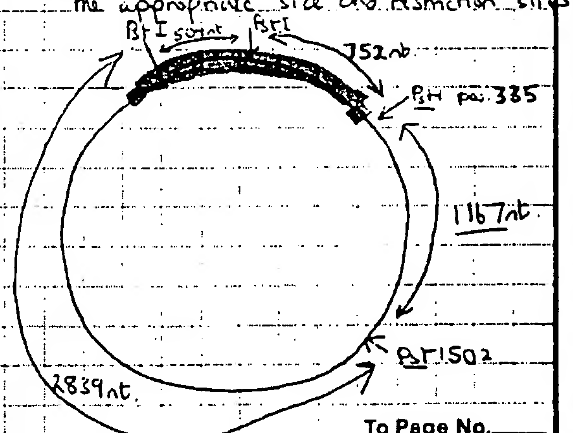
Meanwhile plated out YPH499/5W on YPD agar, to get a stock on agar plates.

Also continued digestion of (11) with Asp718 - ~ 2hrs in 40μl - 1μl Enz.



Lisa's digestions of potential 1.3K clone.

Gel indicates that the 1.3K clone (super (2)) is indeed the correct clone - and has the appropriate size and restriction sites



To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by [signature: David K Clarke]

Project No.

Book No.

TITLE Analysis of double digests Asp718/BstUI on potential 0.9K + 3.5K clones. Read of Neut Assay

From Page No.

Checked growth of yeast cells in broth + plate cultures. The broth looks as though it is growing, and there are very, very small colonies on the plate – things should have improved by tomorrow.

Made a final reading on the Neut assay + Back Titration $TCID_{50}$

-1 -2 -3 B9320 $V_6 DC5 : V_4$ 32°C 10^{-1}

$10^{3.5}$

oops!

The diluted stock which was used in the neutralisation assay contained 5×10^3 pfu ml^{-1} ∴ 100µl contained 5×10^2 pfu

Mouse Number	1/10	1/20	1/40	1/80	1/160
1	0	0	1	4	16
2	0	0	0	5	15
3	0	0	0	1	3
4	0	0	2	6	15
5	0	1	2	4	7
CON					

0 = No plaques visible (1–16 = No of plaques)

Control cell 'monolayers' were wiped out

Will use mouse No 3 for monoclonal production

Prepared a 1% Agarose gel for analysis of

To Page No.

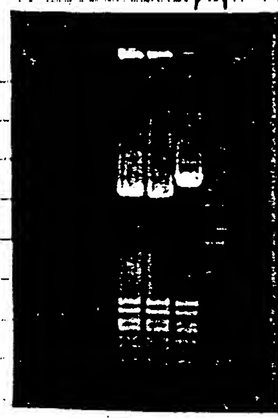
Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kolb		Recorded by David K Clarke	

000400

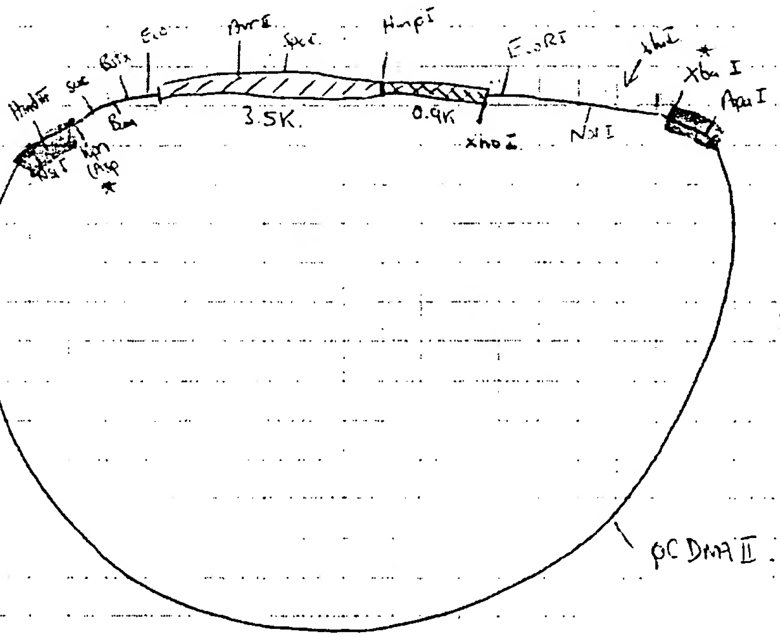
From Page No. 160

Gel shows that clone No (11) is probably the 0.9K + 3.5K clone – will check with further digests.

(11)
1KB ladder



Structure of MCSite of 0.9K + 3.5K in pcII vector



▬ = M.C.S belonging to pcII Vector

▨ = M.C.S. belonging to pCR II.

To Page No.______

Witnessed & Understood by me.	Date	Invented by	Date
Serge W. Kubik		Recorded by David K Clarke	

Project No.
Book No.

TITLE Patent Writing / Yeast cell check

From Page No.

Checked growth of yeast on YPD agar - colonies are quite large, but will leave for another 24hrs, before storing at 4°C

Tidied up Flu T.C Room etc.

Rewrote some of Patent application.

Set up o/n agar plate of 11 (0.9K + 3.5K in pcII)

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Gary W. Keller		Recorded by David K Clarke	

Project No.
TITLE: O/N YEAST CULTURE YPH499 Book No.

From Page No.

Took bacteria (9.9K + 3.5K in pcII) from incubator – it had grown quite poorly as expected – and stored at 4°C. Will set up a mini prep tomorrow for long term storage of this clone in glycerol at -80°C.

Took yeast from 30°C incubator and set up O/N YPH499 in ~2.5ml of YPD broth. – Used a very large loopful of yeast cells to inoculate the O/N culture.

Continued writing Patent material

Temp check on the T.C. Room incubators.

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

[signature]

Recorded by David K Clarke

Project No.____
Book No.____
TITLE Neutralisation assay for MAB I-13. Preparation of Competent Yeast Cells YPH499

From Page No.__

8-45 Inoculated ~100ml of YPD broth with ~1.2ml of O/N culture

Will check O.D at ~1pm or 2pm vs YPD blank

2pm checked O.D 600 of culture - it was ~ 0.531 - will check again ~ 3-30pm

Meanwhile carried out 'Back titration' on diluted (10^{-2}) V_6DCSV_4 32°C 10^{-1} and a virus neutralisation assay using I-13 MAB and a PIV3 HN MAB

MAB Dil^n 1/10 1/20 1/40 1/80 1/160 100µl (~500pfu) into each dil^n of AB

Control - No AB

Meanwhile Kathy shot up Mouse N° 3 with ~300µl of conc. 320 - final boost before harvesting spleen (i.p injection)

Went through Protocol for preparation of competent yeast cells - harvested at 0.8 - 1.0 A_{600} - 2.5K/5mins in 50ml ~~tubes~~ tubes
Resuspended each pellet in 10ml 0.1M LiAc
Respun
Resuspended each pellet in *5ml* 0.1M LiAc and incubated at 30°C for 1hr at 80rpm in small petri dishes
Recentrifuged cells, resuspended in 0.5ml 0.1M LiAc and stored O/N at 4°C.

NB Yeast Cell growth took ~ 8hrs to reach 1.0 A_{600}.

Also: Use YPD broth that has been autoclaved for 15mins only

To Page No.____

Witnessed & Understood by me, [signature] | Date | Invented by | Date
Recorded by David K. Clarke

Plan for assembly of RSV 5' end (L-end)

TITLE: Transformation of Yeast – Li Acetate – No Carrier

Project No.

Book No.

From Page No.

I had forgotten to put O/N 11's (0.9K + 3.5K in pcII) in incubator – set it going and will use it to set up O/N plasmid prep (large scale) for this plasmid

Went through Yeast Transformation protocol – used 50μl of cells/transformation – added 1μg, 100ng, 10ng of pRS416 – plated out cells on Ura⁻ Minimal Medium (after finally resuspending in 100μl of H_2O) – Incubated for 48hs at 30°C

Plan for assembly of Virus 5' end

① A) Digest 3.0K clone with XhoI and PmlI to determine orientation. Once orientation is established, digest with PmlI and phosphatase, then digest with suitable M.C.S enzyme XhoI or Kpn I (Acc718)

② Digest 1.5K clone with Sph and PmlI + (HhaI to remove interfering Vector bands)

Purify the DNA on a gel

③ Digest Bam/Sph clone with BamHI to check orientation; Once orientation is established cut with SphI – phosphatase (using correct conditions), then cut with BamHI and HhaI (to remove Vector DNA) and purify the appropriate band

④ Find conditions for 3-way ligation – use 2:1:2 ratio (molecular) initially, and include some BamHI and either XhoI or KpnI (Acp718) in the ligation to force the 3-way ligation – analyse aliquots on a gel at 5, 10, 20, mins at 37°C — check them buffer for 3-way compatibility!

⑤ ~~Clone~~ Ligate 'best' material into Bam and XhoI or KpnI (Acp718) cut and phosphatased and gel purified Vector.

To Page No. 166

Witnessed & Understood by me, Sergey W. Kohl

Date

Invented by

Date

Recorded by David K Clarke

Project No.______

Book No.______ TITLE ______

From Page No. 165

Used mini prep broth (11) (0.9K + 3.5K in pcII Vector) to inoculate a 250ml broth for preparation of large stock of this plasmid.

Froze down 2 vials of (11) mini prep in glycerol (15%) at -80°C

To Page No.______

Witnessed & Understood by me, Sergey W. Klokh

Date

Invented by

Date

Recorded by David K Clarke

Orientation of 3K and 2.25K clones by Restriction Digest.

Project No.

TITLE:

Book No.

From Page No.

Set up XhoI digestion of 3K clone and Bam HI digestion of 2.25K clone in order to determine orientation of the clones, relative to the M.C.S.

Set up digests in 40µl, with 1.5µl enzyme for ~ 2-3hrs at 37°C.

Phenol Chloroform extracted XhoI digestion of 3K clone and EtOH ppt^ed.

Spun down and set up PmlI digestion — 30µl, 1.5µl Enzyme ~ 1.5hrs 37°C

Prepared a 1% Agarose gel, but left until tomorrow to run out samples.

Lisa ran through plasmid purification for (0.9K + 3.5K in pcII) — Stored in EtOH - will dry down 5.0µl tomorrow and run on the gel

Checked Yeast Transformants — there appears to be some colonies starting on the 1µg transformation plate. Will check again tomorrow. May need to investigate electroporation as a means of transformation - to boost efficiencies.

Went over Monoclonal preparation protocol with Dave S.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
George W. Klh		Recorded by David K. Clarke.	

Project No.______
Book No.______ TITLE Monoclonal Antibody Preparation - harvesting Spleenocytes. Gel analysis of 2.25/3K digests from yesterday

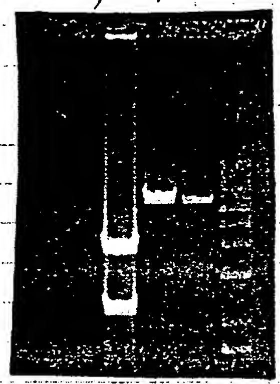
From Page No.__

Checked Yeast transformations - there are 20-30 colonies on the 1µg plate; not yet visible on the 100ng and 10ng plates.

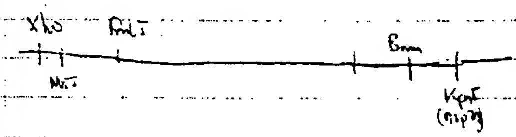
prepared 1% agarose gel and ran out digests from yesterday

1KB ladder / Xho Cut 3K / Xho/PmlI cut 3K / Bam cut 2.25K / Lisa's plasmid prep

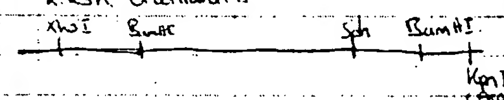
Lisa's plasmid prep did not give anything



3K orientation is :-



2.25K Orientation is :-



(3hr procedure) Went through Monoclonal antibody preparation protocol - Spleen was on the left side of the Mouse, and was very enlarged. Kim demonstrated teasing out the cells with a bent syringe needle. Used RPMI medium, instead of DME medium

Preparative Will digest 3K (150µl) with PmlI - then phosphatase and digest with KpnI and purify 3K DNA.

~8µg (5µl) Will digest 2.25K with SphI - then phosphatase and digest with HhaI/Xho I and purify 2.25K band

will digest 1.5K SphI + PmlI + (HhaI) - to remove vector and purify 1.5K band.

Checked I-B MAB neutralisation assay - No neutralisation was apparent in any of the wells - Virus may have drifted; Virus strain may not be B9320 - MAB may not be active, or may be incorrect.

To Page No.____

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K Clarke

000408

Bulk digestions/Phosphatasing of 3K, 1.5K and 2.25K

TITLE: Restreaking clone Banks

Project No.

Book No.

From Page No.

Prepared 400ml L-Agar (Amp+) - poured ~ 12 plates in order to restreak clone banks

Checked Yeast Transformation again; there are ~ 52 colonies on the 1µg transformation, but none at 100ng or 10ng. Will streak master plate with some of the transformants, in order to do some mini preps.

Set up large scale digestions on 1.5K, 3K, and 2.25K ~ 3hrs at 37°C

3K - pml I in 50µl 5µl Enz

2.25K } SphI in 50µl 5µl Enz each
1.5K }

Lisa is preparing solutions for Yeast Electroporation.

Will phenol/chloroform extract and ppt prior to phosphatasing treatment. Will digest 1.5K with PmlI without phosphatasing (then HhaI).

Talked to Kathy about Monoclonal's:

MAB #	A neut titer	B neut titer
1112	200	5,120
1243	120	5,120

} these two would be possibles for selection against 'B' Helper.

Would also be beneficial to get a Mab(s) against 'G' of A2 which would bind but not neutralize.

Set up PmlI digestion of 1.5K in 50µl - 5µl Enz ~ 3hrs 37°C

Also set up phosphatasing reactions on pmlI cut 3K and SphI cut 2.25K in 100µl with 4µl + 3µl of phosphatase (SAP)

Phenol chloroform extracted all 3K and ppt^ed in EtOH with Glycogen.

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K. Clarke

Project No.______
Book No.______ TITLE Hha I digestions of 2.25K and 1.5K clones to remove vector portion of plasmid

From Page No.__

Checked Yeast and Bacterial Master plates - will leave until later to store at 4°C. The yeast had grown fairly well - so O/N growth on an agar plate should be doable - O/N minis should probably be set up around mid-day for the following morning.

Set up HhaI digests on 2.25K and 1.5K DNAs in 50µl with 5µl of enzyme - incubated at 37°C for ~3hrs.

Phenol Chloroform extracted 2.25K DNA and EtOH ppt[ed]
Stored 1.5K DNA frozen (in 50µl)

Left yeast at 30°C until tomorrow

To Page No.____

Witnessed & Understood by me, [signature] | Date | Invented by | Date

Recorded by David K Clarke

From Page No.

Set up XhoI digestion of 2.25K DNA } in 50µl – 5µl Enzyme
KpnI digestion of 3K DNA }

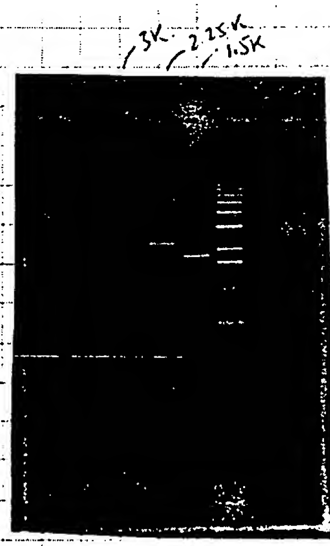
Prepared a 1% gel for analysis of digests (after ~2hrs)

Set up O/N yeast cultures in YPD broth for preparation of Competent cells (Error – tried mini preps of transformants in YPD – Need Drop-out Ura⁻ broth)

Prepared 100ml drop-out broth – autoclaved for 15mins

Set up O/N culture of (0.9K + 3.5K in pcII)

Only the 2.25K fragment has behaved as expected –



1.5K is not migrating correctly (it is too big) Need to cut 1.5K plasmid with
1) PmlI
2) SphI

3K is not migrating correctly (it is too big). Need to cut 3K plasmid with
1) PmlI
2) KpnI
Need +ve PmlI control (λ DNA)

To Page No.

Project No.____ Book No.____ TITLE Preparation of Competent Yeast Cells. Test Digestions of 3K and 1.5K DNAs.

From Page No.__

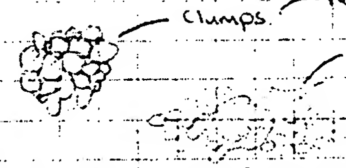
Set up YPH 499 250ml Culture by inoculating with 2.5ml of O/N culture ~9am - Will check OD_{600} ~3pm.

Set up the following digestions.

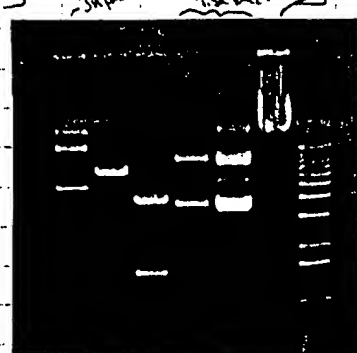
1.5K - pmL 1.5K mini - pmL
1.5K - SphI
3K - pmlI. in 20µl - 1µl Enz in each.
3K - KpnI
λ - PmlI. ~ 2hrs at 37°C

Will analyse on a 1% Agarose gel later

Checked Hybridomas - No contamination so far - there are some clumps of cells which hopefully will grow and expand into hybridomas.



Will check again in a couple of days.



Good; it looks as though PmlI is defective, and is not cutting at all either in λ phage or 1.5 and 3K clones. SphI is cutting okay.

Ordered fresh PmlI.

To Page No.____

Witnessed & Understood by me. Date Invented by Date

Recorded by

Project No.________
TITLE:________ Book No.________

From Page No. 173

Went through preparation of competent yeast cells à la stratagene - stored O/N at 4°C in Li Acetate.

Inoculated 250ml broths (YPD) with 5μl, 50μl and 500μl of O/N YPH499 - will check OD_{600} tomorrow

Set up O/N (0.9K + 3.5K in pcII) in 2 250ml broth Amp+

To Page No.____

Witnessed & Understood by me, Date

[signature]

Invented by Date

Recorded by Daniel K Clarke

Project No.

Book No.

TITLE Preparation of Electrocompetent yeast cells + Transformation
Transformation of LiAc competent cells.
Maxi prep of 4.4K Clone.

From Page No.

Measured OD_{600} of 3 Yeast O/N cultures to be used for preparation of electrocompetent cells – Used culture inoculated with 50 µl of O/N culture – Harvested late and went through transformation protocol for preparation of electrocompetent cells (in the 'Redbook') – Electroporated cells with 1ng, 10ng and 100ng of plasmid – plated out 1/5 of total transformants.

Also carried out LiAcetate ~~electroporation~~ transformation using 10ng, 50ng and 100ng of plasmid – plated out all of transformants

Run through Maxi-plasmid prep with 4.4K plasmid – Yield appeared to be very low – consistent with the toxic nature of this plasmid – will check on a gel tomorrow

Set up 4 O/N Mini Yeast cultures in 3.25ml DOB broth URA⁻

Booked Portugal/Ireland Trip.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

TITLE Yeast Mini-preps ; Checking 4.4K Maxi preps / Fed Hybridomas / O/N 4.4K minis

Project No.

Book No.

From Page No.

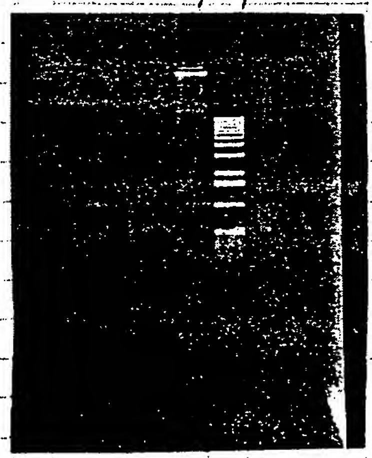
Ran through mini prep extractions for Yeast cells - spun down ~ 3ml of O/N culture and followed stratagene protocol.

Prepared a 1% Agarose gel and loaded ~ 1/5 of the total prep
~ 1/50th of one sample

Ran 6µl of Maxi-prep on a 1% gel - doesn't look as though the extraction worked - may have to purify the DNA from multiple mini preps instead.

4.4K maxi prep

1KB ladder



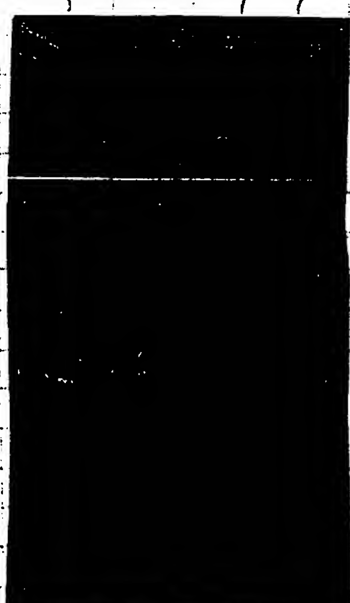
Removed some of hybridoma supernatant from 96 well plates and fed with 100µl of fresh HAT medium.

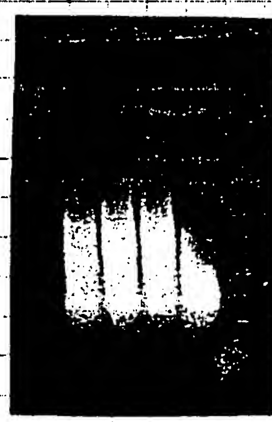
Set up O/N 4.4K Minis (16 in total)

Georges Sample

Yeast Mini prep

pRS416





Plasmid yields from Yeast look very low (or none at all!!) Will Rerun a 2nd gel with some ccc plasmid (pRS416).

To Page No.

Witnessed & Understood by me, | Date | Invented by | Date

[signature]

Recorded by [signature] David K. Clarke

Project No.

Book No.

TITLE Mini preps for 4.4K. Check on PstI. On yeast Mini preps

From Page No.

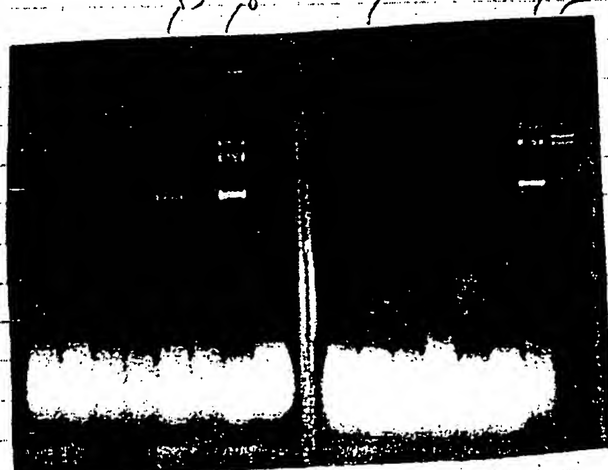
Checked hybridomas — will let grow over the weekend, and then possibly plate out into 24 well plates.

Checked yeast transformants — it looks as though there are numerous transformants, with electroporation being most efficient, giving colonies with 1ng of transforming plasmid. Will leave for another few days.

Carried out Mini prep DNA extractions on 4.4K plasmid x16 — Ran a 10μl sample from each prep (50μl)

Set up PstI digestion of λ phage DNA in 20μl at 37°C for ~1hr

13 8 5 O/N (11) λ phage DNA – PstI cut



Interestingly, it appears that those O/N cultures set up from the original plate gave high yields of plasmid, while those set up from later plates contain very little plasmid — the bacteria are dumping down the N° of plasmid copies per cell

Decided to set up Maxi preps from (8) and (13) minis.
Also streaked some cells from (8) and (13) onto a plate.

Set up 2 x 3.5ml O/N yeast cultures for mini preps tomorrow

To Page No.

Witnessed & Understood by me. Date

Invented by Date

Recorded by

TITLE Yeast Mini preps + Restriction analysis. Maxi - 4.4K prep

Project No.

Book No.

From Page No.

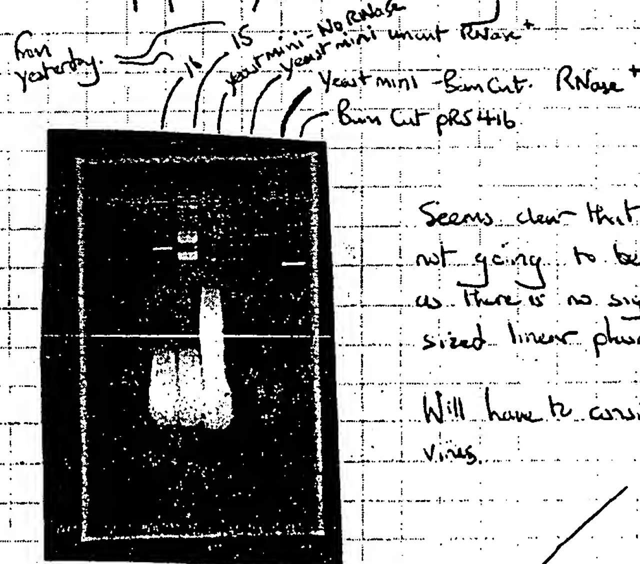
Set up Yeast Mini preps x2 (doubled the amount of Zymolase used in spheroplasting) Lisa went ahead with maxi-prep of ⑧ = potential 4.4K

Set up Bam HI digestion on pRS416 + Yeast mini prep - included some RNase A

Prepared a 1% Agarose gel and ran out samples

LHS / Bam HI cut pRS416 / Bam cut Yeast Mini / Yeast Mini, RNase + / Yeast mini, RNase⁻ /

Also ran out mini preps 15/16 from yesterday.



Seems clear that yeast is not going to be useful to us as there is no sign of the correct sized linear plasmid band.

Will have to consider baculo vines.

To Page No.

Witnessed & Understood by me, [signature] Date

Invented by Date

Recorded by David K Clarke

000417

Project No.______
Book No.______ TITLE PstI digestion of 1.5K and 3K DNAs.

From Page No.__

Set up PstI digestion of 3K plasmid (spun down 150µl of EtOH ppt).
Also PstI digested the existing 1.5K material, which has already been digested with SphI/HhaI.

Digestions were done in ~50µl vols with ~3-5µl enzyme for ~3hrs at 37°c.

Possible solutions to cloning problem.

1). Colony purify 1.3K and/or 0.9K clones and then 'cure' the clone(s) of the plasmid by successive passage in non-selective medium

2) Try cloning 1.3K in Baculovirus transfer vector to see if it is stable in that vector system / transform sure " their host lines.

3). Cloning in λ phage if it will accept 15Kb fragments etc.
A) Will only be useful for in vitro transcription approach.
(see George).

4). Clone in VZV cosmid system. or try cosmid system by itself.
A) May not be useful if have to clone into cosmid first and go through E.coli.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kobe		Recorded by David K. Clarke	

000418

TITLE Gel analysis of PvuI/SphI/HhaI cut 1.5K DNA and PvuI cut 3K DNA. SAP+ of 3K DNA (PvuI cut).

Project No.

Book No.

From Page No.

Prepared a 1% agarose gel for analysis of PvuI cut 3K and 1.5K DNAs.

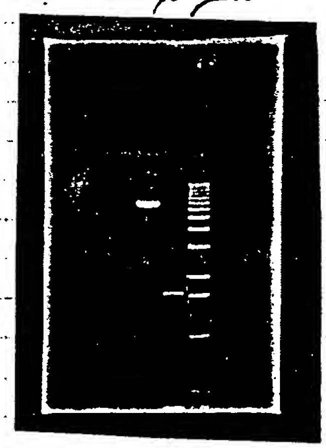
Set up phosphatase RXN on PvuI cut 3K DNA.

in 100µl final vol - adding 4µl phosphatase - then 3µl after the first half hour.

Checked hybridomas - there are definitely some hybridomas growing as slightly enlarged - round groups of cells ("Pearls").

3K PvuI cut.

1.5K PvuI/SphI/HhaI cut.



The 1.5K clone is still too large by ~100b.p.

The 3K PvuI digest looks okay

Will check 1.5K clone with EcoRI and BstXI

EcoRI should give 924 + 610 bands.

BstXI should give ~1600 band.

To Page No.

Witnessed & Understood by me, Serge W. Clark

Date

Invented by

Date

Recorded by David K. Clarke.

000419

Project No.______
Book No.______

TITLE Sorting out 'positive' hybridomas.
Digestion analysis of 1.5K and 3K DNAs
~~Transformation of JC9387 with 1.3K DNA.~~

From Page No.__

Prepared 200ml L-Agar for JC9387 cloning.

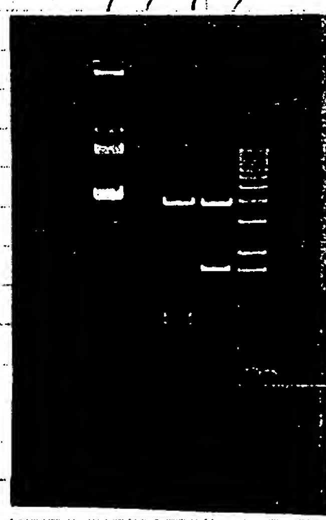
Set up digestions EcoRI cut 1.5K } in 20µl - 1µl Enz - 2hrs at 37°C.
BstXI cut 1.5K }
KpnI cut 3K DNA (PmlI cut + phosphatased) in 50µl
with 5µl Enz ~3hrs at 37°C.

prepared 1% agarose gel for analysis of digests.

Dried down 2µl of 1.3K clone plasmid - resuspended in 5µl H_2O and used 1µl to transform some JC9387 cells.

plated out 100, 10, 5µl aliquots of SOC grown transformants.

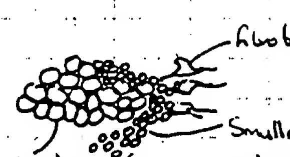
[Will have Lisa digest 4.4K plasmid]



EcoRI and BstXI digestions of 1.5K clone indicate that the 1.5K clone is not quite correct by ≈ 70-100nt & will set up a fresh RT/PCR RXN on Wed.

The 3K PmlI/KpnI digestion looks fine, but there is not much material remaining (~200 ng of the 3K band). Will probably have to repeat these digestions + SAP.

Checked through all six 96 well plates to look for hybridomas, and prepared 2x HAT medium



To Page No.____

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Recorded by David K. Clarke

Gel analysis of PmlI cut 3K DNA and 4.4K DNA.

TITLE

Project No.

Book No.

From Page No.

Checked JC 9387 transformants – it does not look as though there are any white colonies – will leave a few hours more

Also checked streaked out 1.3K clone – there are some single colonies – will pick those later and grow up as mini preps.

Spun down 150 μl of 3K clone and PmlI digested in 50 μl – 5 μl Enz ~3hrs 37°C.

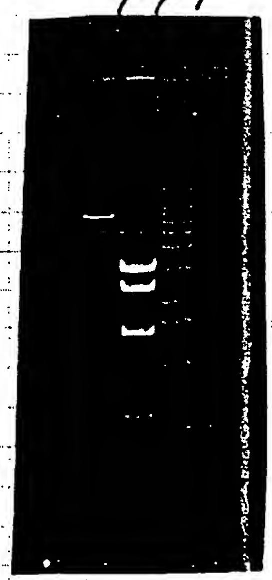
Lisa is doing some diagnostic cuts on 4.4K plasmid (EcoRI)

Checked hybridomas – Removed some media and then transferred (with 5-10 up-and-down pipettings) the remaining media too a fresh 96 well plate; refed the original wells and incubated as pairs at 37°C

Prepared fresh 14mM Methyl mercury and 1.85M β-Mercaptoethanol, for RT/PCR RXN.

Set up O/N '3K sure' so Lisa could use to do a Maxi prep later in the week.

also set up eight 1.3K minis from individual colonies as a prelude to passaging in the absence of antibiotic



PmlI digest shows ~ 100-200ng of DNA ∴ ~ 2-4μg total

4.4K digest looks good; all 4 predicted insert bands are present adding up to the correct 4.4K size.

To Page No.

Witnessed & Understood by me, Serge W. Klein | Date

Invented by | Date

Recorded by David K Clarke

Project No.

Book No.

TITLE RT/PCR for 1.5K region Sph/Pml. Phosphatasing of PmlI cut 3K DNA.

From Page No.

Carried out phosphatasing of PmlI cut 3K DNA - did RXN

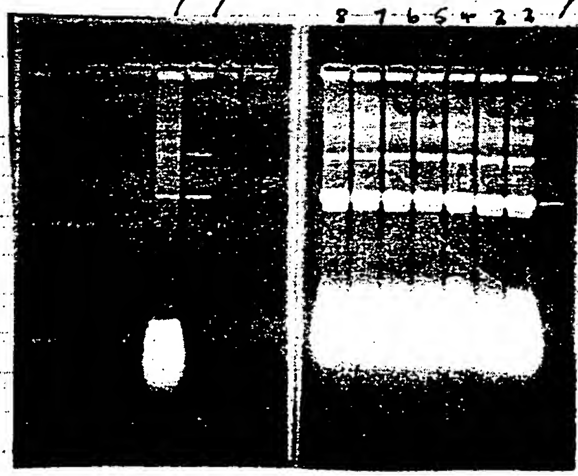
in 100µl final vol. with 5µl SAP, 30mins, 37°C then another 2µl SAP, 37°C 30 mins.

Phenol extracted and EtOH ppt^ed.

Carried out mini preps on 3K mini + 7 x 1.3K minis - ran 2 x 1% agarose

gels to analyse the products

Will set up a 3K MAXI prep with 'test' 3K O/N culture.



Will try to passage all seven clones (1.3K) in L-B broth minus Amp (used 5µl inoculation.). Will check each mini prep at 5-passage intervals.

Set up RT/PCR RXN in an attempt to recover 1.5K region.

Did a 'Hot-start' for Taq RXN (added Taq at 80°C

Spun down 0.8ml of genomic RNA
Dissolved in 3µl H_2O / 3µl Sph → Pml primer
Heated to 83°C for 5mins → Ice.
Added 0.6µl 14mM Meth Merc. - 4mins R.Temp
Then added 0.6µl 2-mercaptoethanol - 4mins R.Temp.
Added 4µl of this to an RT RXN in 20µl final vol;
with µl RTase.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Gary W. Keble		Recorded by David K. Clarke	

000422

continued Passage of 1.3K clones without
Analysis of 1.5K RT/PCR PRODUCTS.

TITLE ~~Digestion of 3K DNA with KpnI + gel analysis.~~ Project No.______ Book No.______

From Page No.__

Prepared 1% agarose for analysis of RT/PCR product - loaded $1/10^{th}$ of total along with 1Kb ladder.

Stored O/N 1.3K passages (2-7) as glycerol stocks at -70°C (150μl glycerol + 850μl O/N culture).

Set up KpnI digestion of 3K DNA (PmlI cut + SAP treated). In 50μl final vol. 5μl Enz. 2-3hrs at 37°C.

1.5K RT/PCR products.
1KB ladder

Tomorrow will run out - Long excise the larger DNA band and elute the smaller DNA band onto NA45 paper

Again, can see a doublet in the 1.5 - 1.7K region

The smaller DNA fragment is the one that I want

Chloroform extracted and EtOH ppt^ed with a couple of μl of glycogen

prepared a fresh batch of NA45 paper.

Ran out a sample of PmlI/KpnI SAP+ 3K DNA along with a little of the Maxi prep DNA (Lisa's).

Called YEAST expert (Tims Supervisor) to see if cloning in Yeast would be possible; He said no.

Prepared some fresh NA45 paper and stored at 4°C.

To Page No._____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Klein (signature)		Recorded by David K. Clarke (signature)	

Project No.

Book No.

TITLE

From Page No. 183.

Good. Gel shows perfectly cut
3K DNA ~ 30ng in the appropriate band
20x30 = 600ng of correct DNA
Will pool with earlier material

The ccc Maxi prep. also looks good.

To Page No.

Witnessed & Understood by me.	Date	Invented by	Date
[signature]		Recorded by David K. Clarke	

Project No.

TITLE: Gel purification of 1.5K RT/PCR product. Book No.

From Page No.

Prepared 1% Agarose gel for gel purification of RT/PCR (1.5K) products.

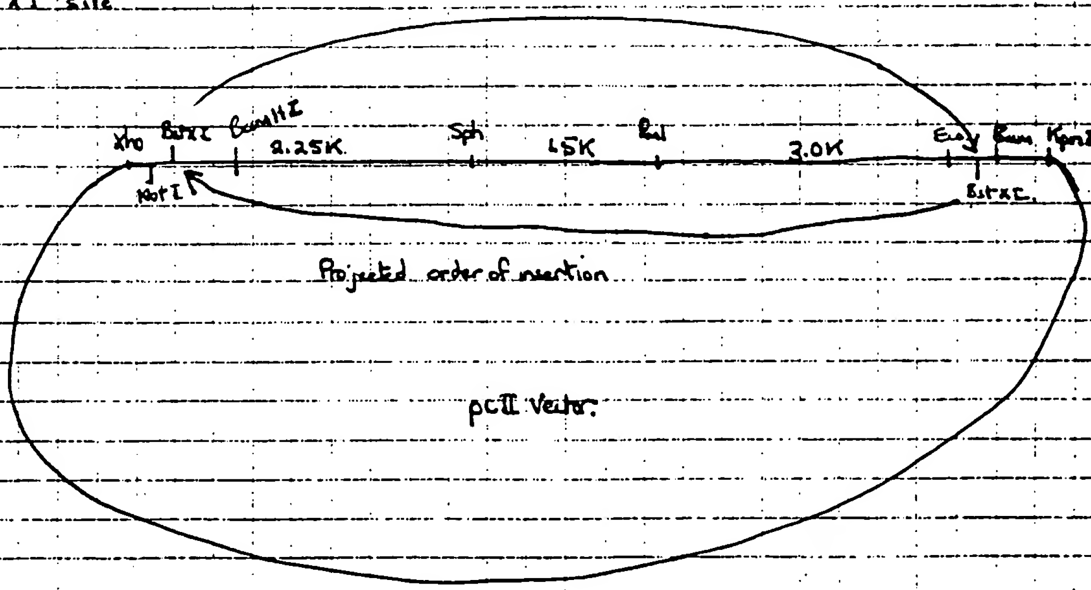
Run bromophenol blue off the bottom to maximise separation of duplex.

Checked hybridomas – difficult to tell if the hybridomas are growing or not – Will leave them over the weekend.

Electroeluted 1.5K DNA onto NA45 paper (Excised the 1.65K band first – before electroeluting) Phenol chloroform extracted and EtOH ppt'd with some glycogen.

Lisa continued with the passage of 1.3K clones in ~~att~~ the absence of Antibiotic – I will keep them going over the weekend.

If 1.5K + 3K + 2.25K clone is obtained (≡ 6.75K), then may need to flip it around at the BstXI site.



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Witnessed & Understood by me, Date Invented by Date

Recorded by

Project No.

Book No.

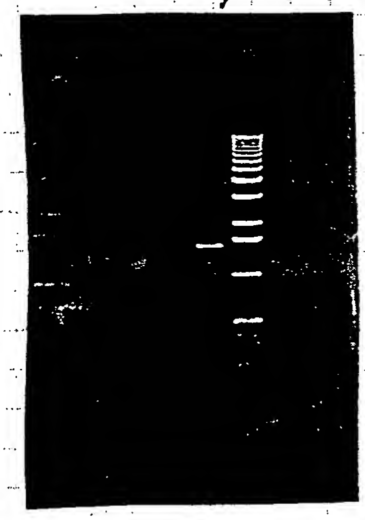
TITLE Analysis of 1.5K RT/PCR DNA - Gel purified. Passage of 1.3K clones in absence of antibiotic.

From Page No.

Prepared a 1% agarose gel for analysis of 1.5K gel purified RT/PCR product - Run ⅓ of total purified DNA alongside 1KB marker.

P4 Continued 1.3K clone passages 2-8, using a 5µl inoculum into Antibiotic minus L-8.

Gel purified 1.5K RT/PCR product.



Good! gel shows that the 1.5K band is enriched ~10 fold over the 1.65K contaminating band. Will ligate 4µl with ~100ng of PCR II vector O/N Sun → MON.

To Page No.

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K. Clarke

O/N ligation of gel purified 1.5K RT/PCR product with pCR II vector.

TITLE: Passage of 1.3K bacterial clones

Project No.______

Book No.______

From Page No.__

Took off O/N 1.3K cultures and stored a sample of each at -70°C in 15% glycerol. - Continued passage with a 5µl inoculum from each to fresh L-B.

Checked hybridomas - it looks as though some of them are growing in the sub-culture 96-well plates - will check again tomorrow, and remove a little medium, then replace it with HAT+ medium containing O.P.I. (likewise in original 96 well plates).

Set up O/N ligations at 12°C between purified 1.5K DNA and pCR II vector.

2µl x10 buffer
4µl of DNA (1.5K ~ 25ng).
3µl of Vector ~ 75ng.
10µl H_2O
1µl Enz
20µl

set up a control minus 1.5K DNA.

To Page No.____

Witnessed & Understood by me, Serge W. Klee

Date

Invented by

Date

Recorded by David K Clarke.

Project No.______ Book No.______

TITLE Transformation of 'SURE' cells with 1.3K + pCR II Ligation Mixture.

From Page No.__

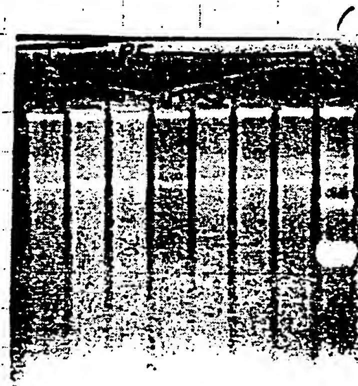
Prepared L-agar (200ml) for transformations, to be done later today.

Lisa went ahead with mini preps and gel analysis of 1.3K clones.

— prepared RPMI medium + O.P.I. (100ml) for growth of hybridomas
Removed some of old medium and replaced with 100µl of O.P.I medium.
Transformed 100µl of 'SURE' cells with 5µl of O/N ligation mixture — heat shocked at 42°C for 45 secs. Incubated in 900µl of SOC for 1hr to allow phenotypic expression. (with frequent shaking). Prepared IPTG/X-Gal Amp$^+$ Agar plates.

5* Plated out 3x200µl on Amp$^+$ plates, and 1x200µl for CON. → O/N 37°C

con. 1.3K



It would appear that virtually all of the plasmid has been lost after 5 passages — although there are some traces in 2, 3, 6, 7, 8.

1) Will proceed with population passages through P_{10}.
2) Will check back on $P_1 \to P_2$, $P_2 \to P_3$ and $P_3 \to P_4$ (mini preps).
3) If little plasmid is present on some of the earlier passages — will streak out to single colonies — then check them on ~~on~~ amp$^+$/amp$^-$ replicate plates then recheck the amp^S colonies for absence of plasmid.
4) Then prepare competent cells from amp^S clone(s) and transform with 1.3K DNA — if stable transformants are generated then the exp. is successful!

To Page No.____

Witnessed & Understood by me. Date | Invented by | Date

Siegfried W. Kohl

Recorded by David K. Clarke.

O/N Minis/Masters of potential 1.5K clones.

TITLE Mini prep analysis of 1.3K clones (for loss of plasmid)

Project No.

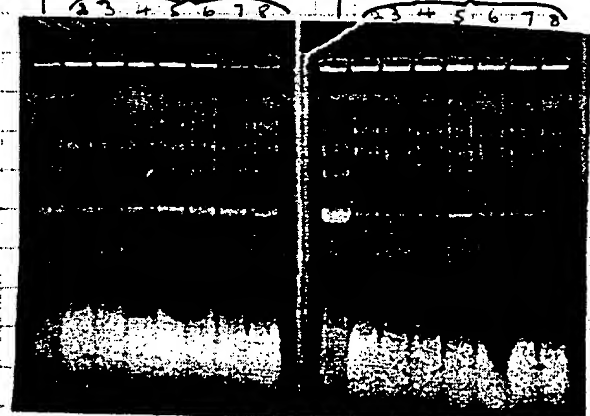
Book No.

From Page No.

Checked transformants — there are numerous white colonies on plates where 1.5K DNA was lygated into pCR II vector. Control plate shows only blue colonies - seems like there should be some transformants - will streak out ~ 36 and set up 10 O/N mini preps.

Prepared 3 x 1% agarose for analysis of mini preps $P_2 \to P_3 \to P_4$ (to find where 1.3K plasmid is first lost from bacterial clones).

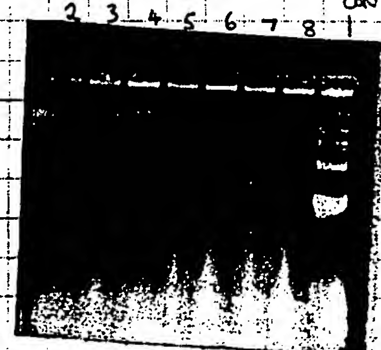
CON. P_2 CON. P_3



There is a clear gradation of plasmid loss from P_2 through P_4 and P_5.

Set up O/N minis for potential 1.5K clones.

Should be able to streak out P_4 colonies on Amp⁻ L-B agar (for 2-7), then look at 3-4 colonies from each by mini prep - after replicate plating on Amp+/- plates



P_4

To Page No.

Witnessed & Understood by me. [signature]

Date

Invented by

Date

Recorded by [signature: David K. Clarke]

Project No.

Book No.

TITLE Potential 1.5K ~~maxi~~ clone mini preps and digestion + gel analysis. Growth of potential 1.3K plasmid minus clones.

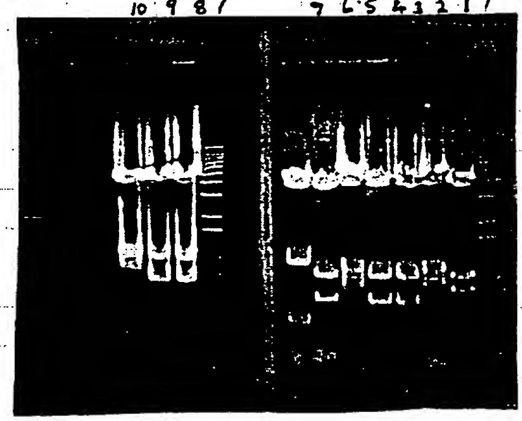
From Page No.

Carried out 10 mini preps on potential 1.5K clones — set up EcoRI digests in 20µl with 1µl Enz + RNase for ~ 2hrs at 37°C.

prepared 2 x 1% agarose gels for analysis of digestion products.

Prepared 1litre of L-Agar for streaking out potential 'plasmid-minus' bacteria (1.3K clones 2-7). Streaked each of these clones out to single colonies (from P_4 populations).

10 9 8 1KB ladder 7 6 5 4 3 2 1 1KB ladder



3, 8, and 9 also look as though they contain inserts of the correct size. Will check maxis with BstXI, and EcoRI cuts

Selected clones 4 and 6 to be grown up as Maxi preps in 250ml O/N cultures.

Streaked out 1.3K clones 2-7 to single colonies — will replicate plate 10 colonies from each clone on $Amp^{+/-}$ plates to check for loss of the 1.3K plasmid.

To Page No.

Witnessed & Understood by me. Serge W. Kible

Date

Invented by

Date

Recorded by David K. Clarke

Replicate plates of potential 1.3K clones
Maxi preps of 1.5K clones (4)+(6) and EcoRI/BstXI
TITLE Digestion analysis of these clones. Project No. Book No.

From Page No.

Checked streaked out 1.3K clones - will pick up to 10 clones from each plate for Amp^{r}- selection.

Meanwhile checked all 6 96-well plates for evidence of hybridoma cell growth - there are about 40-50 hybridomas in total - will check them again on SAT. or SUN. and sub-culture into 24 well plates

Streaked out 1.3K clones and grew O/N at 37°C.

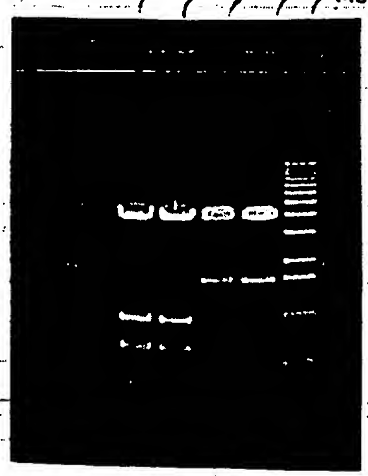
Lisa carried out Maxi preps on clones (4) and (6) for 1.5K region -

EcoRI and BstXI digestions on both (4) and (6) were set up in 20μl of final vol - Used 1μl of DNA, 1μl of Enz - 1.5hrs at 37° and 55°C respectively.

Prepared 1% agarose gel for analysis of digestion products.

Lisa continued with passage of 1.3K clones to get rid of plasmid.

6 EcoRI
4. EcoRI
6 ~~EcoRI~~
4 ~~EcoRI~~ BstXI
1KB ladder



Good! Looks like the 1.5K clones are both correct.

The BstXI frag should be 1,573 (and is).
The EcoRI frags. also look to be the correct size.

To Page No.

Witnessed & Understood by me, Date Invented by Date

Recorded by David K. Clarke

Project No.______ Book No.______ TITLE Bulk digestion of 1.5K plasmid (4) SphI and PstI.

From Page No.__

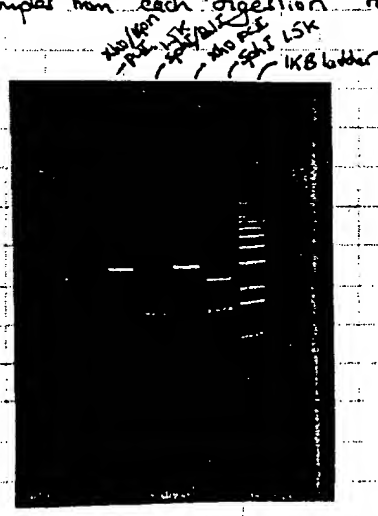
Checked growth of replicate plate '1.5K' clones — Good! it appears that all the clones are Amp sensitive which would indicate they have lost their plasmid.

Set up SphI digestion on 1.5K DNA. — used 5µl of DNA ~ 5µg in a 50µl digest with 5µl enzyme ~2hrs at 37°c.

Also set up XhoI digestion of 3µg of pcII DNA in 50µl with 2µl Enz - 2hrs at 37°c.

Phenol chloroform extracted and EtOH ppt^ed. Then set up PstI digestion of SphI digested 1.5K and KpnI digestion of XhoI digested pcII DNA.

Retained small samples from each digestion for gel analysis.

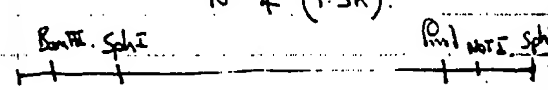


Prepared 1Litre of fresh Hybridoma Medium containing O.P.I. but Minus HAT (to be added separately later).

Lisa filter sterilised

Good! looks as though double digests have worked. Will need to do a final HhaI digestion on 1.5K DNA to remove Vector DNA from the 1.5K band.

No. 4 (1.5K).



SphI pos. 368 in Vector
NotI " 353

To Page No.___

Witnessed & Understood by me, | Date | Invented by | Date

Recorded by David K. Clarke.

Title. HgaI digestion of 1.5K DNA
Phosphatasing of pcII DNA.
Gel purification of 2.25 and 3K DNAs.

Set up HhaI digestion on 1.5K DNA to remove contaminating Vector DNA. - in 50µl final vol - 5µl Enz ~ 2-3hrs at 37°C.

Set up Phosphatase RXN on Xho/KpnI digested pcII vector DNA - in 30µl with 2µl of SAP, 30mins - then 1µl more SAP. Will freeze both until tomorrow, and then gel purify.

Prepared a 1% agarose gel, and ran out 2.25K DNA and 3.0K DNA for gel purification onto NA45 paper.

Checked hybridomas, and 'earmarked' those to be subcultured into 24-well plates tomorrow. Also found a few 'new' hybridomas.

Completed electroelution and phenol/chloroform extracted; then precipitated the DNA with a little glycogen.

Recorded/Invented by: David K. Clarke

Date:

[signature]

Title.

Prepared a 1% agarose gel for Electroelution of pEll DNA (sm p+ etc) and 1.5K DNA (SphI - PvuI - HhaI cut).

Sub-cultured hybridoma cells into 24 well plates (23 in total) - those that looked well-grown only - Pipetted up and down fairly vigorously, then transferred to the 24 well plate (containing 1ml growth medium OPI+, HAT+).

Proceeded then to stain agarose gel, and continued with electroelution onto NA45 paper - phenol extraction and precipitation with a small quantity of glycogen.

Set up O/N cultures of 'cured' 1.3K clones - Set up 4 minis (all No. 4 of 10) from series 2, 3, 4, 5, - will make competent cells from each tomorrow.

Recorded/Invented by:- David K. Clarke

Date:-

George W. Klehle

DATE DUE	BORROWER'S NAME	ROOM NUMBER

[redacted]

Book 3

LABORATORY NOTEBOOK

DAVID CLARKE

000437

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Q4M

NOTEBOOK NO. 74

ISSUED TO David K. Clarke

ON

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000439

INSTRUCTIONS

1. **The primary purpose of this notebook is to protect your and the Company's Patent-Rights by keeping records of all original work in a form acceptable as evidence if any legal conflict arises.**

2. • When starting a page, enter the title, project number, and book number.
   • Use ink for permanence -- avoid pencil.
   • Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
   • Avoid making notes on loose paper to be recopied.
   • Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.

3. • Give a complete account of your experiments and the results, both positive and negative, including your observations.
   • Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
   • Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.

4. • After entering your data, sign and date the entries.
   • Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
   • Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.

5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.

6. This notebook and its contents are the exclusive property of the Company. It is confidential and the contents are not to be disclosed to anyone unless authorized by the Company. You must return it when completed, upon request, or upon termination of employment. It should be kept in a protected place. **If loss occurs, notify your supervisor immediately, and make a written report describing the circumstances of the loss.**

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Preparation of Competent cells 2-5

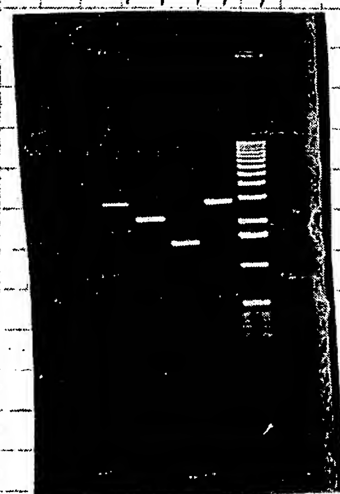
TITLE Gel analysis of NA45 purified 3K, 2.25K, 1.5K and pcII DNAs — Project No. — Book No. — 1

From Page No.

Inoculated 100ml L-B broths with 0.5ml of O/N culture (from O/N minis of 2, 3, 4, 5 - 'cured' 1.3K clones) - to prepare competent cells.

Meanwhile prepared 300ml L-Agar, Amp+ for plating out transformants

Spun down gel purified 3K, 2.25K, and 1.5K + pcII DNA and resuspended in 20µl H_2O - then loaded 4µl (1/5) for gel analysis.



Will use 4µl of pcII Vector + 4µl 3K, and 2µl 2.25K - in 20µl ligat^n vol. 1µl Enz, and will sample at 10 mins, 30 mins and 1hr.

Will attempt to ligate pcII vector (2.4K) with 3K and 2.25K Tomorrow.

pcII DNA is SAP+ Kpn/Xho.

2.25K is XhoI cut, and SphI cut with SphI site SAP+.

3K is PmlI cut with SAP+ at that site and KpnI cut

Continued with transformations - Dried down 3µl of 1.3K DNA, and resuspended in 20µl H_2O - used 1µl to transform 2, 3, 4, 5, competent cells - plated out 200µl of 1ml final vol. (after 'rescue' of cells) onto X-gal/IPTG Amp+ Agar.

Lisa set up O/N minis on 1.5K 3, 4, 6, 8, 9 to recheck and freeze down 1.5K clones.

Checked hybridoma cells - the look mostly good - will refeed older cells tomorrow, and

To Page No.

Witnessed & Understood by me, [signature] | Date | Invented by | Date

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Project No.______

Book No.______

TITLE Trial ligation of 3K and 2.25K into pcII vector
O/N Minis of potential 1.3K transformants

From Page No.__

Checked bacterial transformants - two of the four transformations appear to have worked - with a small n° of white colonies visible - the other two plates do not appear to have any colonies - but will incubate all four for a few hours more.

Set up pcII, 3K, and 2.25K ligation.

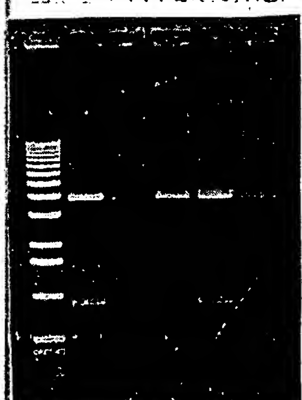
Prepared a 1% agarose gel for analysis of ligation products.

Lisa did mini preps on potential 1.5K clones.

4µl 3K DNA.
2µl 2.25K DNA.
4µl pcII DNA
1µl T_4 ligase
2µl x10 buffer
7µl H_2O
20µl • 37°C for 10mins, 30mins, 1hr

Sampled 6µl at

Fed older hybridoma cells with fresh O.P.I + HAT medium.



Lisa's mini preps of 1.5K clones - to be frozen down as glycerol stocks

1hr 30min 10mins 1KB ladder

None of these bands are of the correct size to be ~8K required. Will proceed with double ligation i.e. 2.25K + pcII DNA. (2µl each in 20µl final vol.)

It may be better to put clones together one at a time i.e. get a 1.5K clone in a suitable orientation and and add it onto the 3K clone - then add the 2.25K. Should be able to flip the 1.5K DNA with BstXI digestion + religation.

Set up O/N Mini preps of 8 potential 1.3K clones from 'LATE 5' cells.

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Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kbl		Recorded by David K. Clarke.	

Mini prep analysis of 1.3K clones from 'LATE 5'

TITLE Ligation of pcII DNA + 2.25K DNA

Project No.

Book No.

From Page No.

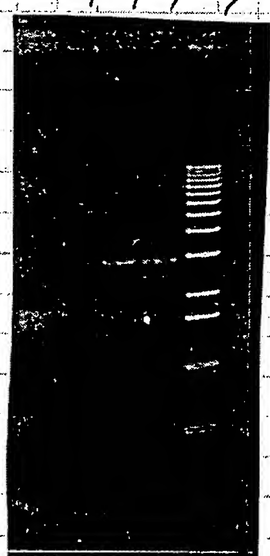
Ran through 8 mini-preps of potential 1.5K clones from 'LATE 5'

Prepared 1% agarose gels for analysis of EcoRI digests of mini prep DNAs.

Lisa carried out mini preps on 1.5K clones to determine orientation of insert.

Set up a 2-way ligation between pcII DNA and the 2.25K clone

1hr 30min 10min 1KB ladder

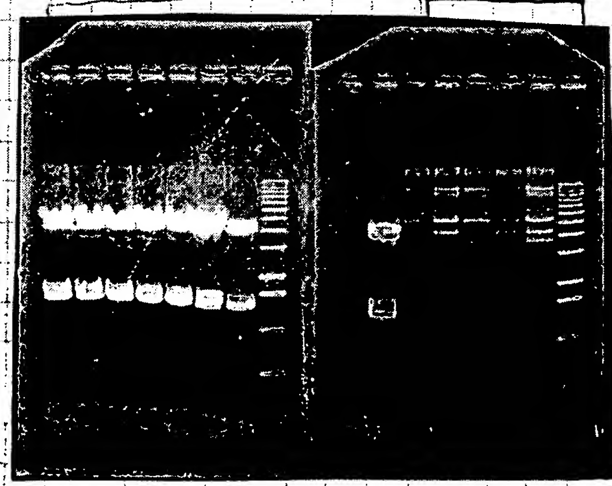


2µl 2.25K DNA
2µl pcII DNA.
2µl x10 RXN buffer.
13µl H_2O.
1µl Enz.
20µl

Sampled at 10mins, 30mins, 1hr at 37°C.
Loaded/ran gel for analysis.

1.3K clones look good.

XbaI analysis of 1.5K clones.



ccc plasmid

There are no clear candidates for the 5.25K expected band. May abandon this approach.

1051 + MCS 6bps = ~1100bp.

⊗ looks like the one I want based on partial digestion products. - Will set up an O/N culture tonight to check by HindIII digest tomorrow.

To Page No.

Witnessed & Understood by me,

Date

Invented by

Date

Recorded by David K. Clarke

Project No.______

Book No.______

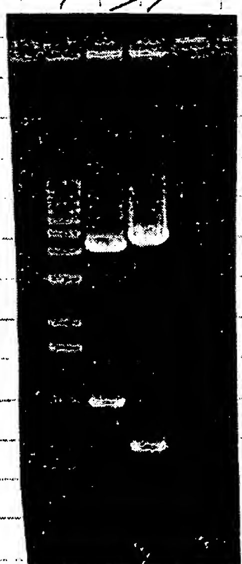
TITLE Bulk digestion of 1.3K clone, to provide insert + Vector (EcoRI) and gel purification.
Determination of orientation of 1.5K clones

From Page No.__

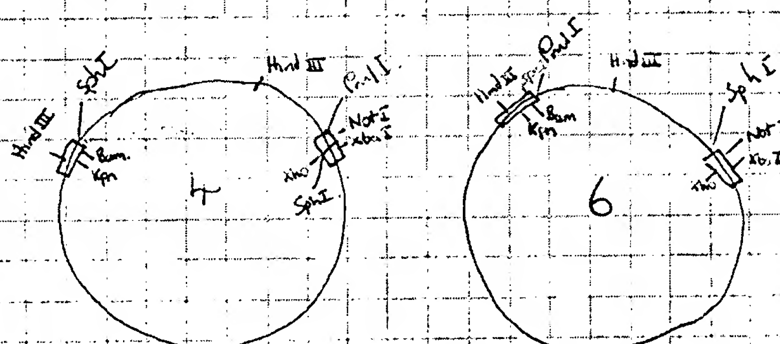
Lisa is doing Hind III digestion on (4) + (6) 1.5K clones to do a final determination of orientation.

Set up bulk digestion of 1.3K to prepare insert + vector to flip the 1.3K insert around in the EcoRI site. Can use a BstXI digestion to determine the orientation of the resulting clones.

λ/8 ladder 1.5K clones (Hind III cut).



Good, it appears that (4) and (6) are in opposite orientations.



Clone Orientations.

Completed electroelution of 1.3K DNA (Vector + insert) - Phenol Cloroform extracted and EtOH ppt'd with 2μl of glycogen.

Set up O/N 'LATE 5' - to freeze down glycerol stock.

Checked hybridomas - they appear to be growing fairly well for the most part; Will decide how to proceed tomorrow.

To Page No.______

Witnessed & Understood by me, Serge W. Kh[illegible]

Date

Invented by

Date

Recorded by Daniel K. Clarke

Gel analysis of purified 1.3K and Vector DNAs.
Restreaking of clone banks.

TITLE Large scale digestions of 1.5K/2.25K with HhaI.

Project No.______
Book No.______

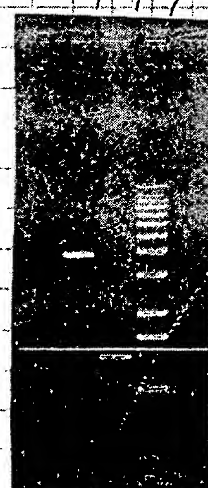
From Page No.__

Prepared 500ml L-Agar (Amp^+) to restreak clone banks.

Spun down gel purified '1.3K and Vector' DNA and resuspended in 8μl H_2O – analysed 2μl of each on a gel, along with 1KB ladder.

10.30am – Set up 'large-scale' digestions of 2.25K and 1.5K DNA with HhaI – Used 6μl of enzyme in 60μl final vol for 4-6hrs. Then phenol/Cloroform extracted and EtOH ppted.

Poured Agar plates – Dried.



Checked Hybridomas – Sub-cultured 3 more hybridomas into 24 well plates – Checked those already growing – will collect ~50μl of supernatant from the most densly populated plates tomorrow, and sub-culture into 25cm² Flasks.

Good – both the insert and Vector have been purified in reasonable amounts. Will proceed with phosphatasing of Vector component (± 400ng).

Set up phosphatase rxn on Vector component in 20μl, using 1μl Enzyme, followed by a 2nd 1μl aliquot after ~30mins at 37°C – proceeded to phenol/chloroform extract and EtOH ppt.

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Witnessed & Understood by me.	Date	Invented by	Date
Serge W. Kolb		Recorded by David K. Clarke	

Project No.

Book No.

TITLE SphI digestion of 2.25K and 1.5K DNAs.

From Page No.

(Thought: consider subcloning 2.25/1.5 into pcII Vector).

Set up SphI digestions on 2.25K and 1.5K DNAs (HhaI digested).

Did RXNs in 60µl final vol. – used 6µl enzyme for ~3hrs at 37°C; then phenol chloroform extracted and EtOH ppt^ed.

Collected hybridoma supernatant from most densely populated 24 well plates, before sub-culturing into 25cm² bottles. (Collected 500µl from each hybridoma – six in all were sub-cultured today.

Removed Bacterial master plates from 37°C and stored at 4°C – all had grown adequately.

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Xho I digestion of 2.25K DNA
SpeI digestion of 1.5K DNA } Gel analysis.

TITLE O/N ligation of 1.3K DNA + VECTOR

Project No.____
Book No.____

From Page No.__

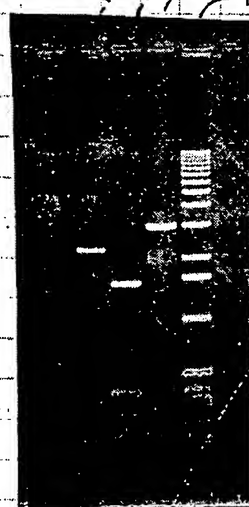
Set up digestions of 2.25K DNA and 1.5K DNA. Each in 50µl final vol. with XhoI for 2.25K and SpeI for 1.5K. Used 5µl Enzyme in each case. Set up a control for SpeI (ordered some new SpeI).

Lisa transformed some DH5α with pcII DNA to prepare a maxi prep of it.

Prepared a 1% agarose gel for final analysis of digests - will make a 2nd gel of gel purification of fragments later to day.

Fed hybridomas in 96well plates in the hopes of coaxing them to grow out a little more.

cut 2.25K/Xho
Hind/Xho
Hind/Xho/spe cut 1.5K (6)
SpeI cut pcII (con) — Enzyme appears to be working at a reasonable efficiency
1KB ladder



Set up O/N digestion of pcII DNA with XhoI - in 30µl final vol. using 3µl enzyme - covered to reduce evap/condensation.

Checked hybridomas - they all look pretty good

It appears that all the digestions have worked appropriately - prepared another 1% agarose gel and ran out large scale digestions - there should be ~

50 x 100 ng = 5µg of 1.5K DNA
50 x 50 " 2.5µg - 3µg of 2.25K DNA

Completed Gel purification of DNA bands, Phenol/chloroform extracted and EtOH ppt^ed

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Witnessed & Understood by me, Date Invented by Date

Recorded by David K. Clarke

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Project No. ______

Book No. ______ TITLE Analysis of gel purified 1.5K, 2.25K DNA
Transformation of 'Late 5' with (1.3K+vector)
Phosphatasing of 1.5K DNA

From Page No. ___

Inoculated 100ml L-B with 0.5ml O/N 'LATE 5' - for competent cell production

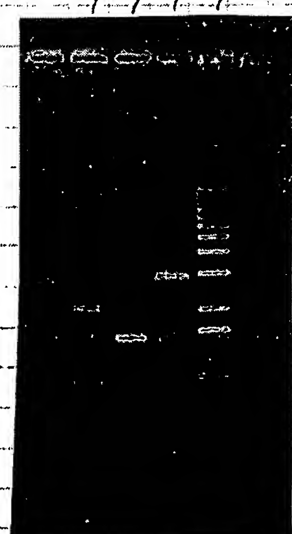
Prepared a 1% agarose gel for analysis of gel purified 1.5K and 2.25K gel purified DNAs - will look at ½μl - ¼th of total. Will also look at XhoI digested pcII DNA - 1μl of 3μl

Prepared 500ml L-Agar for plates to be used in transformation. - added Amp - poured - dried. Did transformation using 10μl of ligation mixture (of 20μl). Heat-shocked 42°C for 45secs, rescued for 1hr and plated out on X-gal⁺/IPTG⁺ plates.

Carried out phosphatasing of XhoI cut pcDNA II - in 20μl final vol, - used 2×1μl of enzyme in a 1hr total incubation. Phenol extracted and EtOH ppt^ed.

Carried out sub-culture of hybridoma cells into 25cm² flasks. - stored 500μl of each supernatant for assay in neutralization test.

2.25K DNA - Gel purified XhoI/SphI
1.5K DNA Gel purified XhoI/SpeI
XhoI cut pcII DNA.
1KB LADDER



Gel to analyse amount of DNA obtained from gel purification.

There appears to be ~ 100ng ×40 = 4μg of 1.5K DNA.
~ 2μg of 2.25K DNA.

Set up phosphatase RxN on all of 1.5K DNA in 50μl final vol.
5μl ×10
40μl containing DNA
3μl Enz + 2μl Enz after ~30mins - then phenol/Chlor. extracted and EtOH ppt^ed.

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Witnessed & Understood by me, Date

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Recorded by David K Clarke

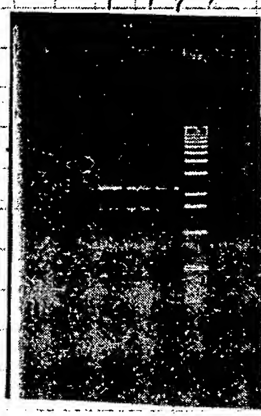
O/N minis of potential 1.3K clones
Digestion of phosphatased pcII DNA with Spe I. - Gel purification. Project No.
TITLE Trial ligation of 1.5K DNA and 2.25K DNA. Book No.

From Page No.

Set up SpeI digestion of XhoI cut/SAP⁺ pcII DNA.
In 30µl final vol - with 3µl Enzyme.
Prepared a 1% agarose gel for gel purification of pcII DNA.

Proceeded to Electroelute and phenol extract the SpeI/XhoI+SAP⁺ pcII DNA.
EtOH ppt^{ed} with 1µl of glycogen.

Meanwhile set up ligation between 1.5K and 2.25K DNA.



Can see a very low level of 3.75K product, which I require.

2µl 1.5K DNA
2µl 2.25K DNA
3µl x10 XhoI buffer
3µl x10 BSA
3µl 10mM ATP
2µl Enz (XhoI)
1µl DNA Ligase
14µl H_2O
30µl

Will repeat this ligation tomorrow but use double the amount of each DNA used, and leave O/N at 37°C - will then add a little extra XhoI the following morning.

Checked the hybridoma cells - they are all doing okay - removed 500µl of medium from the remaining 24 well plates and added 500µl of fresh medium. Will subculture 96well plates into 24 well plates tomorrow.

Set up a scheme for Lisa to carry out a "back-up cloning" procedure for the addition of the 1.5K to the 2.25K clone.

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Recorded by

Serge W. Kolb

David K Clarke

Project No.

Book No.

TITLE Mini preps + BstXI digestion + gel analysis of potential 1.3K clones
Ligation of 1.5K + 2.25K O/N.
Sub culture of hybridomas

From Page No.

Carried out mini prep DNA extractions on potential 1.3K clones – and then set up BstXI digestions, in an attempt to diagnose clones in either orientation;
(however BstXI is not a good choice – so repeated extractions and digested with PstI).

Also ran out Gel purified pcII DNA (XhoI/speI cut and partially SAP'd) to check amount of DNA

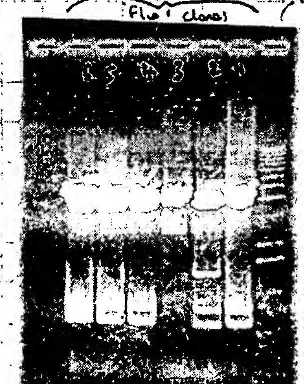
pcII DNA.
1KB ladder

There is ~ 30ng/μl – total
20×30 = 600ng of DNA.
remaining

proceeded to set up ligation between 2.25K DNA and 1.5K DNA in the presence of XhoI.

4μl 1.5K DNA
4μl 2.25K DNA.
3μl x10 XhoI buff.
3μl x10 BSA.
3μl 10mM ATP
2μl XhoI.
1μl DNA lig.
10μl H_2O
30μl O/N 37°C.

1-6 BstXI digests of potential 1.3K Flo' clones
1KB ladder.



Carried out a 2nd mini prep extraction and set up PstI digests.

N°2 looks like a Head-to-tail doublet
N°3 looks like reclosed vector

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Witnessed & Understood by me, Date

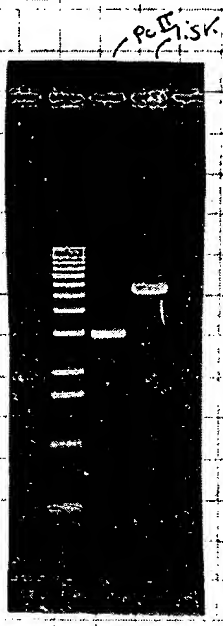
Invented by Date

Recorded by David K. Clarke.

TITLE

Project No.
Book No.

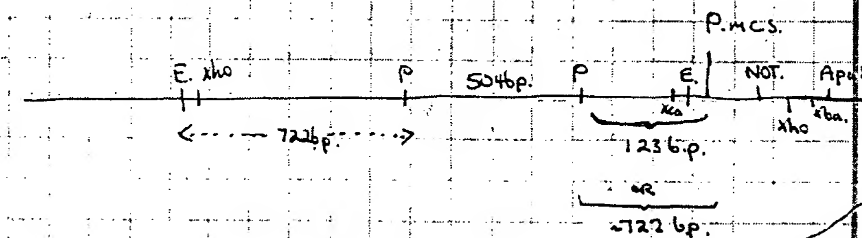
From Page No. 10



Lisa's Apa I digest of pcII DNA and (4) 1.5K DNA.
Both appeared to work fairly well.

Sub-cultured hybridomas in 96-well plates into 24-well plates (11 in total) – Pipetted fairly vigorously and transferred into 1ml fresh O.P.I medium + HAT.

Pst I digestion of 1.3K gives a 504 b.p. internal (in 1.5K) fragment.



Set up O/N Master plate of 1.3K clones

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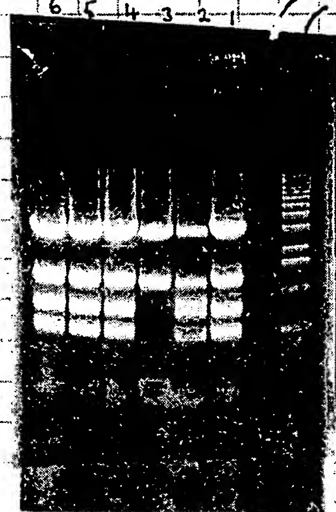
Project No.______

Book No.______

TITLE Analysis of PstI digests of potential 1.3K 'Flip' clones
Analysis of 1.5 + 2.25K Ligation/XhoI Rxns.
Ligation of 1.5K + 2.25K into pcII DNA vector

From Page No.___

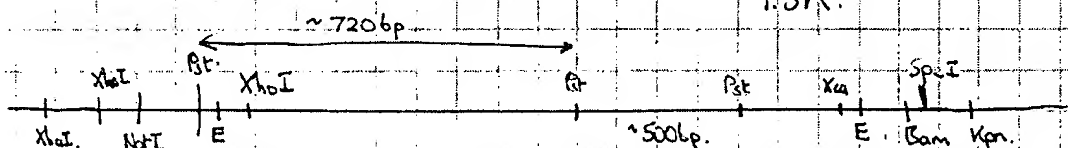
Ran out all six potential 1.3K clones - PstI digested - also ran out 6µl (1/5th) of 2.25/1.5K ligation/xho mixture to check ligation pattern.



There are some high M.W. DNAs generated by the 1.5K + 2.25K ligation - Phenol/Chlr. extracted the remaining RXN and set up a ligation with some pcII vector (XhoI - SnP Spe I cut.)

All of the 1.3K containing clones are in the same approximate orientation (even the one containing the head to tail insert).

The PstI digest pattern indicates the following orientation within the M.C.S.



Page 161 of BK 2.

May be able to cut 4.4K with XhoI and Xba, then purify the large DNA, and ligate to Spe I/XhoI cut (1.3K) SpeI is compatible with XbaI
(Will get Lisa to grow up more 13K DNA.)

Will also need to prepare electro competent 'Late S' cells.

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Witnessed & Understood by me. Date Invented by Date

Recorded by

From Page No. 12

Set up an O/N ligation between ligated 1.5K and 2.25K into XhoI/speI — Digested pcII DNA.

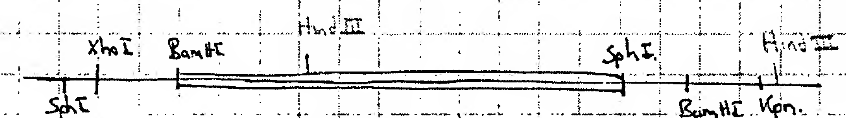
Have 10-20ng total of correct sized DNA band will ligate with ~ 200ng of pcII vector

10µl H_2O
7µl pcII DNA.
2µl x10
1µl Enz
20µl 37°C O/N.

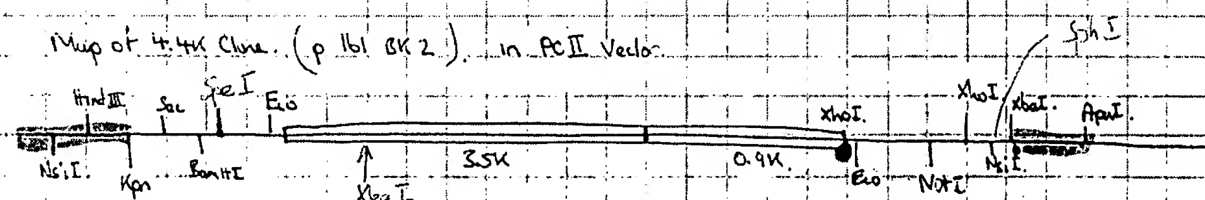
Prepared Amp+ Agar plates for transformations tomorrow. - dried and stored at 4°C.

Checked hybridoma cells

Map for 2.25K Clone (p 168 BK 2)



Map of 4.4K Clone (p 161 BK 2) in PCII Vector



Subcultured hybridomas (RSV) 1—6 into 25cm² flasks in a 1→2 split

Hybridomas 8, 10 and 11 look good for freezing - will freeze down tomorrow if they still look good.

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Project No.
Book No.
TITLE Tranformation of 'SURE' cells with 1.5K + 2.25K in pcII. Digestion of 4.4K + 1.3K. Hybridomas 7-13 frozen

From Page No.

Set up restriction digest of 4.4K DNA. – XbaI.
5μg of DNA in a 50μl RXN, with 4μl Enzyme for ~3hrs at 37°C.
Also set up SpeI digest of 1.3K DNA (50μl spun down from stock) in 50μl with 4μl of enzyme for ~3hrs at 37°C

Then phenol extracted and EtOH ppt^ed Saved 1/50^th each for gel analysis

Carried out transformation of 'SURE' cells with ligated 1.5K and 2.25K into pcII vector – prepared X-gal / IPTG / Amp+ plates.

Checked Hybridomas – will freeze down 7 through 13 today and probably do 1-6 tomorrow. Will also do some more sub-culturing tomorrow.

Used 'freezing medium' according to Kathy's protocol (+Gln)

To Page No.

Witnessed & Understood by me, Date | Invented by | Date

Steve W. Kohler

Recorded by David K Clarke

ON minis of potential 1.5K + 2.25K in PCII.
XhoI digestion of 4.4K + 1.3K DNAs.

TITLE: Hybridoma subculture/Freezing

Project No.____

Book No.____

From Page No.__

Checked 1.5K + 2.25K in PCII transformants - there are quite a few whites
- will set up ON minis + Master -

Will cut with ~~EcoRI~~ Hind III.

Set up XhoI digestion of 1.3K DNA in 30μl with 2μl of enzyme for ~4hrs at 37°C
also set up XhoI digestion of 4.4K with 5μl of enzyme for ~4hrs at 37°C.

Will Phenol extract and EtOH ppt 4.4K DNA prior to phosphatasing.
Will freeze 1.3 K. (removing 1μl for gel analysis) prior to gel purification.

Subcultured remaining hybridomas in 96 well plates into 24 well plates;
also subcultured hybridomas 14 through 26 into 25cm² Flask, from 24 well plates - saved 500μl of supernatant from each for assay purposes.

Also froze down two more hybridomas from 25cm² Flask (1 + 6)

Set up o/N 1.3K culture for Lisa to use as inoculum for Maxi prep.

Also set up a large batch of mini preps to check for 1.5K + 2.25K clone
34 in total.

Selected an RSV paper for Ying to cover in RA Journal club.

To Page No.____

Witnessed & Understood by me, Serge W. Kibble

Date

Invented by

Date

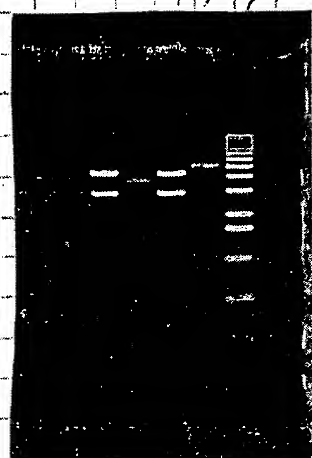
Recorded by David K Clarke

Project No.
Book No.
TITLE Mini preps of potential 1.5K + 2.25K in PCII clones
Gel analysis of 1.3K and 2.25K Digests from weekend
Hybridoma Freezing etc.

From Page No.

Started mini preps for potential 1.5K + 2.25K in PCII - ran through 16 mini preps - Lisa did 18 more.

Loaded 1.3K and 4.4K digests on a 1% agarose gel



Unfortunately there is an 'invisible' XbaI site in the 4.4K clone - so will be unable to use this strategy.

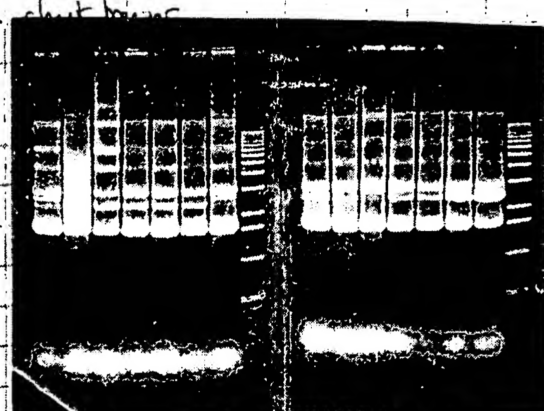
Instead will digest 1.3K DNA with XbaI and XhoI and 4.4K with SpeI and XhoI - will phosphatase 1.3K DNA and Ligate in the 4.4K Insert.

Will make electrocompetent cells to facilitate this process.

Set up HindIII digests of potential 1.5K + 2.25K in pcII
Run out on 1% agarose gel to see if any clones are correct.

Set up XbaI digestion of 1.3K DNA in 30µl with 2µl Enzyme for ~3hrs at 37°C
Set up SpeI digestion of 4.4K DNA in 50µl with 4µl Enzyme for ~3hrs at 37°C.
Will save 1µl of each for gel analysis

Checked hybridomas - Froze down N° 4 in 1ml freezing medium - stored previously frozen hybridomas in chest freezer



Doesn't look as though any of the clones are giving me the DNA bands which I need.

Need to see a 1.9K band to indicate correct clone and a 600bp band.

To Page No.

Witnessed & Understood by me, Date
Serge W. Kitth

Invented by Date

Recorded by
David K. Clarke

TITLE XhoI digestion of 1.3K and 4.4K + Gel analysis
O/N 'Late 5' for preparation of electro competent cells.

Project No.

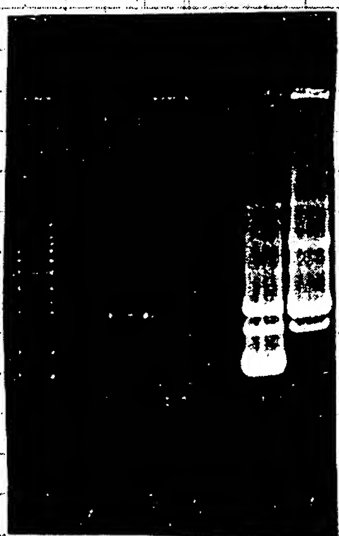
Book No.

From Page No.

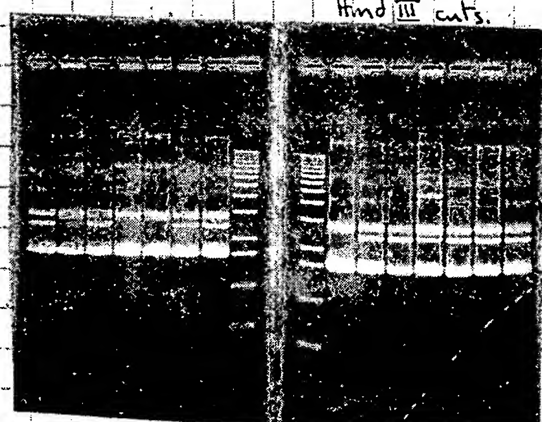
Lisa continued analysis of mini-preps (see below)
Lisa is continuing with Bam HI digests of pcII/2.25K.
Lisa is doing a maxi prep on the 1.3K clone.

SphI cut pcII. SphI cut 2.25K

Set up XhoI digestions on 1.3K and 4.4K DNAs.
1.3K in 30µl - 3µl Enz
4.4K in 50µl - 5µl Enz ~ 4hrs at 37°C

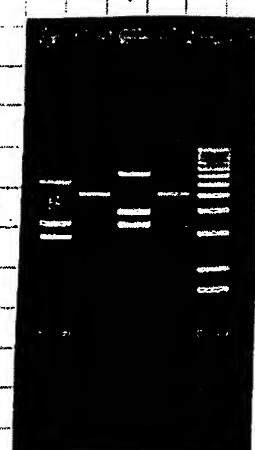


Hind III cuts.



Analysis of remaining 1.5K + 2.25K in pcII clones.
None of the clones look as though they are what I need.

Ran samples from XbaI cut 1.3K, XbaI/Xho cut 1.3, SpeI cut 4.4K, and SpeI/XhoI cut 4.4K DNA.



Gel shows there is an additional SpeI site in the 4.4K - invalidating this approach.

Froze down hybridoma N° 2.

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by [signature]

Project No.______

Book No.______

TITLE Preparation and storage of electrocompetent cells derived from Late 5' clones.

From Page No.__

Inoculated 1Litre of L-broth with O/N Late 5 to prepare electrocompetent cells.

Grow to an OD_{600} of 0.5 - then chilled in ice water for 15mins and proceeded with preparation according to George's protocol.

Froze down in ~100μl aliquots at -90°C. in Box with

Devised a plan for uniting the 1.3K clone and the 4.4K clone without disturbing the orientation of the 1.3K clone with respect to the LacZ promoter etc.

Will use pBluescript II SK(+) from Stratagene.

(A) 1) Cut 4.4K with XhoI and BamHI. → Gel purify 4.4K frag.

2). Bam/Xho cut (SK^+) Phosphatase and gel purify

3). Ligate both together and transform electrocompetent Late 5 cells.

(B) 1) Cut 1.3K DNA with XhoI and KpnI (Asp.) → Gel purify.

2). Cut 'A.3' above with KpnI (Asp.) and XhoI - phosphatase → Gel purify

3) Ligate 1.3K into 'A3'

Checked in on hybridomas - froze down N°5 - stored N°2 with others at -80°C

Will freeze down a large batch tomorrow.

To Page No._____

Witnessed & Understood by me, Serge W. Kobble

Date

Invented by

Date

Recorded by David K Clarke

TITLE Freeze-down of hybridomas - Subculture where necessary. Planning   Project No.____   Book No.____

From Page No.__

Subcultured 27-32 from ~~Goo~~ 24 well plates into 25cm^2 flasks - froze down supers.
Also froze down 10 more hybridomas from 25cm^2 flasks.

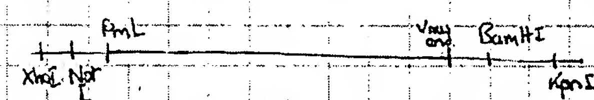
Lisas' gel of 1.3K DNA cut with EcoRI looks good.

A) The 2.25K fragment also looks good - Bam/Sph/HhaI cut. It will be purified and ligated into SphI/Bam HI cut phosphatased pcII DNA

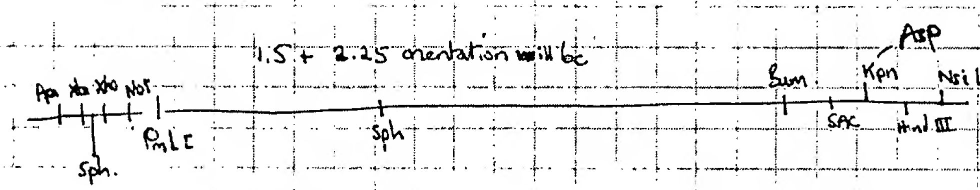
B) Then grown up and purified - then cut with ~~ApaI~~ and SphI and phosphatased and gel purified.

C) Then gel purify SphI/~~ApaI~~ HhaI cut (4) 1.5K DNA and ligate into (B) above - find correct orientation by mini prep.

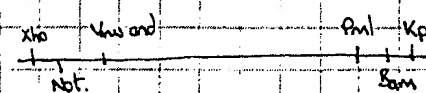
3K orientation is



1.5 + 2.25 orientation will be



(1). Can flip the 3K to give



Then ligate the PmL-~~NotI~~ XhoI frag into Pml/XhoI cut (1.5 + 2.25K)

OR

(2) Transfer the Bam/XhoI frag of 3K clone into Bam HI XhoI cut Bluescript II SK(+) then ligate in Pml/~~Kpn~~ Asp cut (1.5+2.25) into pmlI/~~Kpn~~ Asp I 3K + Bluescript

To Page No.____

Witnessed & Understood by me, Sag W. Killer   Date   Invented by   Date
Recorded by David K. Clarke

20

Project No.

Book No.

TITLE Electrotransformation of Late '5' with pcII DNA 4.4K, and 1.3K DNA's

From Page No.

Prepared 300ml of L-Agar, Amp+ and poured plates. Added IPTG and X-gal for colour screening.

Carried out electroporation of Late '5' cells using 1ng of plasmid in ~30µl of competent cells - pcII DNA, 4.4K DNA, 1.3K DNA.

plated 200µl of 1ml for each x 2.

Helped Lisa with gel purification of 2.25K and pcII DNAs which had been cut with SphI and Bam HI. Phenol chloroform extracted and precipitated with 1µl of glycogen.

Checked in on hybridoma - noted those which needed to be fed, sub-cultured etc.

planned out Bluescript/4.4K digestions - will use 5µg of each in 50µl vol. with 5µl enzyme ~3hs at 37°c

To Page No.

Witnessed & Understood by me, Sage W. Kimble

Date

Invented by

Date

Recorded by David K. Clarke.

Xho I digestion of 4.4K and SK+(Bluescript) DNAs

TITLE: Subculture of hybridoma cells.

Project No.____

Book No.____

From Page No.__

Checked transformants — there are quite a large number of transformants on all three plates ; most on the pcII DNA plate - least on the 4.4K plate indicating that the efficiency may be size related. Will pick 4 colonies from each of 4.4K and 1.8K for mini preps to check plasmid integrity.

Set up XhoI/ digests of both 4.4K and Bluescript DNAs. in 50μl using 5μg of DNA and 5μl of enzyme (~100u) 1-15pm → 4-15pm.

Checked hybridomas — froze down those which were well grown in 25cm² flasks - sub-cultured hybridomas from 24-well plates into 25cm² flasks. (total of 5 - #33 to #37).

phenol chloroform extracted and EtOH ppt^ed XhoI digests - saved 1/50th of each for gel analysis.

To Page No.____

Witnessed & Understood by me, Serge W. Kerlula | Date | Invented by | Date

Recorded by David K. Clarke.

Project No.______

Book No.______

TITLE BamHI Digestion of 4.4K and SK^+ DNAs.
Gel analysis of XhoI digests of 4.4K and SK^+ DNAs.

From Page No.__

Set up Bam HI digestions of XhoI digested SK^+ and 4.4K DNAs
– in 50µl with 5µl of enzyme (100u) for 3hrs at 37°c.

Meanwhile ran a gel to analyse XhoI digests of SK^+ and 4.4K from yesterday.

Checked in on hybridomas – froze down another batch.

Checked gel of XhoI cut SK^+ and 4.4K – Have Run out of film – but digest worked good with both DNA's linearised and giving the expected bands. Thankfully!

Phenol cloroform extracted SK^+ Bam/Xho cut DNA – EtOH ppt^ed.

Saved 1µl from 4.4K/SK^+ Bam digests for gel analysis.

To Page No.____

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kubala			
		Recorded by David K. Clarke.	

TITLE Phosphatasing of SK+ vector. Gel analysis of XhoI/Bam Cut SK+ and 4.4K DNAs.

Project No.______

Book No.______

From Page No.___

Set up phosphatase RxN on SK+ XhoI/Bam HI cut DNA. In 50µl

with 2 + 2.5µl of Enzyme over a ~1hr incubation at 37°C. Then froze down prior to gel purification tomorrow along with Bam/Xho 4.4K DNA.

Prepared a 1% agarose gel for analysis of Bam/Xho Digests of SK+ and 4.4K. (1/50th of total).

Also ran Lisa's Double digests SphI/Bam HI of gel purified DNA to quantitate (1µl of 20µl loaded for each).

Checked in on hybridomas – will need to subculture some from 24 well plates into 25cm² flasks; will also need to freeze down one or two perhaps.

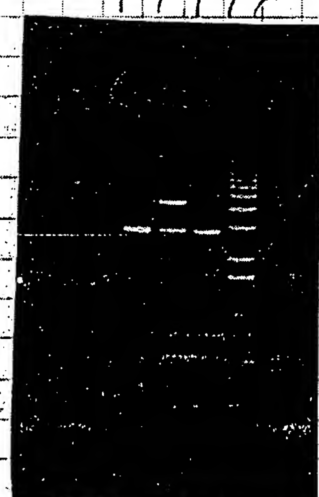
2.25 sph/Bam.

pUI sph/Bam

4.4K Xho/Bam

SK+ Xho/Bam HI

1KB ladder



2.25K has migrated a little unusually and is in low abundance (~10ng µl⁻¹).

Will rerun a gel with 5µl of sample

Will get Lisa to ligate 2µl vector with 10µl insert 2.25K.

SK+ and 4.4K look fine

To Page No._____

Witnessed & Understood by me, Sigi W. Kuhle

Date

Invented by

Date

Recorded by David K. Clarke

Project No.______
Book No.______
TITLE Gel analysis of purified 2.25K DNA (Again!)
Gel purification of 4.4K and SK+ DNAs.

From Page No.__

Ran a 1% agarose gel to recheck the 2.25K gel purified fragment.

Also ran a 1% agarose gel to purify the 4.4K and and SK+ DNA (Xho/Bam digested).

Electroeluted, phenol/cloroform extracted and EtOH ppt^ed (with 1µl of glycogen).

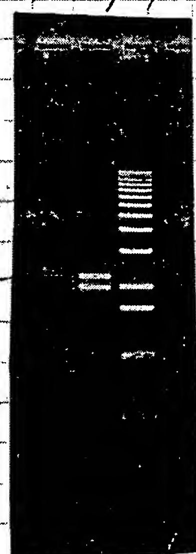
Stored away previously frozen hybridomas and replaced Isopropanol in cell-freezing containers.

Froze down two more Hybridoma cell lines.,

Also passaged hybridomas #'s 38, 39, 40 into 25cm² flasks.

And further split remaining 24 well plate cells into mirrored wells to create more even monolayers.

Purified 2.25K DNA.
1KB Ladder.



2.25K band still looks fine.

There is a 2nd smaller DNA species in the 2.25K (purified DNA); this was most probably generated through nicking activity of HhaI followed by phenol cloroform extraction (acidic; may have resulted in some breakage).

Lisa went ahead with the ligation + Cin.
5µl 2.25K DNA
2µl Vector pcII
2µl x10 buffer
1µl Enz.
H_2O to 20µl O/N R. temp.

Lisa set up O/N Minis for Electroporated transformations with 4.4K, 1.3K and pcII

To Page No.____

Witnessed & Understood by me. Serge W. Kenlich | Date | Invented by | Date

Recorded by David K. Clapho

TITLE Gel analysis of gel purified 4.4K and SK+ DNA.
O/N ligations of SK+ and 4.4K DNA.

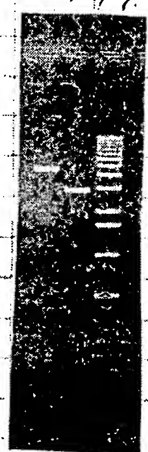
Project No.

Book No.

From Page No.

Prepared a 1% agarose gel and loaded 1μl of 4.4K (gel purified) and SK+ DNA (gel purified) ÷ of 20μl total for each.

Lisa went ahead with the mini prep DNA extractions of 4.4K, 1.3K and pcII (Electroporation clones) - also set up EcoRI digests of these DNAs.



Good both the SK+ and 4.4K gel purified DNAs look good and are abundant; ~ 100ng μl^{-1}

Will Ligate 4μl SK+ with 3μl 4.4K O/N at R.Temp.

3μl SK.
4μl 4.4
2μl x10 + CON SK+ only
10μl H_2O.
1μl Enz.
20μl.

Checked in on Hybridomas - froze down one more #37

Stored Lisa's EcoRI digests of mini preps (4.4K, 1.3K, pcII) at -20°C

To Page No.

Witnessed & Understood by me, Gary W. Kuchel | Date | Invented by | Date

Recorded by David K. Clarke.

Project No. ______
Book No. ______ TITLE Transformations with 4.4K in SK+ Vector using 'SURE' cells and 'Late 5' cells.

From Page No. __

Prepared 300ml L-Agar - for transformations - will use both electrocompetent and 'SURE' cells to get a comparative transformation efficiency

Spun down EtoH p.t.ed Ligations - rinsed and resuspended in 6µl H_2O
Used 2µl to transform 'SURE' cells (from Con. + Test). ~3:30 plate out.

Also used 2µl to transform Electrocompetent cells (Late 5's) 3:45 plate out.

ORDERED
'Sure' cells and - Stratagene.
'SAP' - USB

* see reference binder #074A

1KB ladder 1.3K 4.4K ItII

Gel showing Mini preps from Electroporated 1.3K, 4.4K, ItII. It looks good; all the plasmids are amplified without significant modification.

Checked in on hybridomas - froze down #34 and 35

[redacted]

To Page No. 27

Witnessed & Understood by me, Date

Invented by Date

Recorded by David K. Clarke.

TITLE Digest of 1.3K DNA with KpnI and Isoschizomer Asp 718. Gel analysis of digests.

Project No.

Book No.

From Page No.

Set up restriction digests of 1.3K DNA with both KpnI and Asp 718, which is an isoschizomer of KpnI, on 10μg of DNA (separately for each enzyme)

Did each digest in 50μl, with 5μl Enzyme, for ~ 4hrs at 37°C

Ran 1/50th of each on a 1% agarose gel

Lisa carried out mini preps to determine if any clones contained the 2.25K insert.

Wrote out corrections for Patent Application → Luann Cserr.

Phenol/chloroform extracted KpnI and Asp 718 digestions and EtOH ppt^{ed}.

Checked hybridomas → Subcultured 41, 42 and 43 into 25cm^2 flasks. Stored 500μl of each supernatant at -70°C.

Froze down 24, 27.

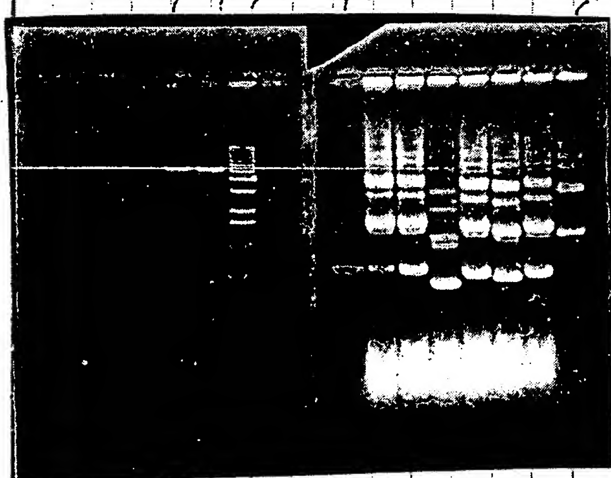
KpnI cut 1.3K

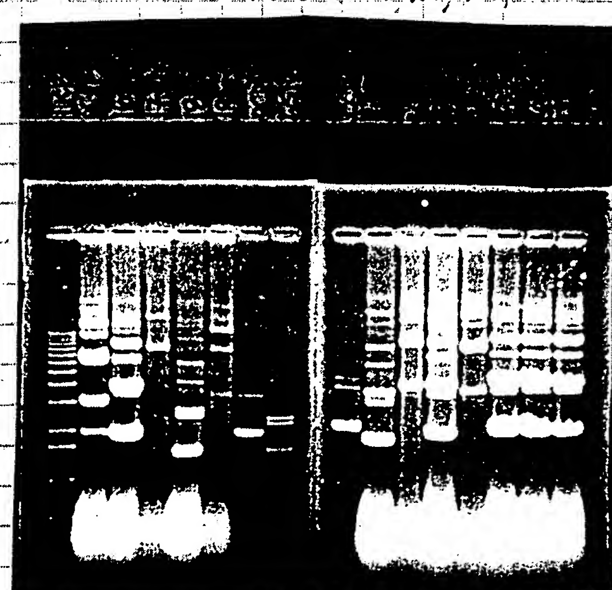
Asp cut 1.3K

1KB ladder

cont. R.I.

14





#s 2, 4, 14, 15 and 19 look promising

Will phenol chloroform extract - ppt and digest with Hind III.

ge No.

Witnessed & Understood by me, Serge W. Kulik | Date | Invented by | Date

Recorded by David K. Clarke

Project No._______
Book No._______

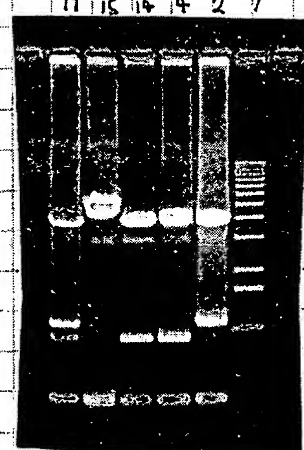
TITLE XhoI digestion of Asp718/Kpn cut 1.3K DNA.
HindIII digestion of Lisa's minis - pot. 2.25K clones.
O/N minis of pot. 4.4K clones in SK+.

From Page No.__

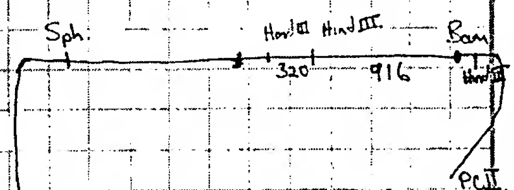
Set up XhoI digestions on both KpnI and Asp718 digested 1.3K DNA.
- in 50μl with 5μl of enzyme for 3-4hrs (~3-4.5pm off)

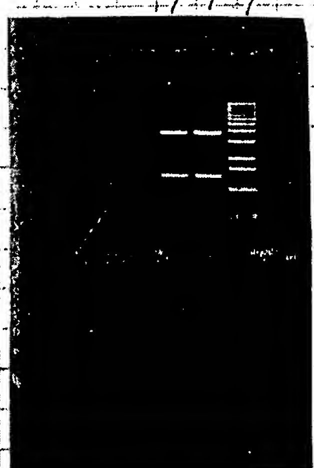
Also phenol cloroform extracted Lisa's potential 2.25K minis - EtOH ppt'ed at -70°C for 30mins the set up HindIII digestions in 20μl with 1.5μl enzyme and 2μl RNase A (off at 3-15pm).

Meanwhile checked hybridomas. froze down 4 more hybridomas



Gel shows that 4 and 14 are the ones I want (i.e. 2.25K in pcII). Will set up O/Ns for Maxi preps.





This gel indicates both digests have worked well, and indicate there is about 2-3μg of the 1.3K insert

To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kimble		Recorded by David K Clarke	

Poster/Patent work. O/N MAXI PREPS of 4.4K in SK+ Project No.______

TITLE Mini preps of potential 4.4K clones Book No.______

From Page No.___

Carried out Mini-prep DNA extraction on ten potential 4.4K clones (SURE) Lisa did Electroporated clones. Will do EcoRI digestions on these DNAs later. – Run 1% Agarose gels to analyse digests.

Prepared a 1% agarose gel and run out Asp718/Xho cut 1.3K DNA for gel purification.

Lisa is doing maxi preps on 4 and 14.

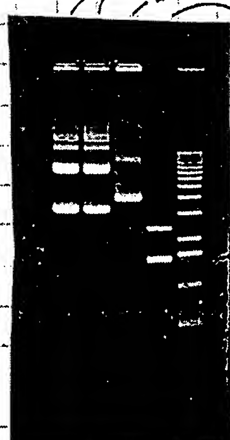
Set up SphI digestion of 1.5K DNA in 50μl (Used 10μl of 1.5K (4) stock). Used 5μl Enzyme for 3-4hrs – run a 1μl sample after ~2hrs to check digestion efficiency.

Electro eluted the 1.3K DNA – phenol extracted and EtOH ppted – Will check 1/20 on a gel tomorrow.

2.25K in pcrII gel

2.25K in pcRII

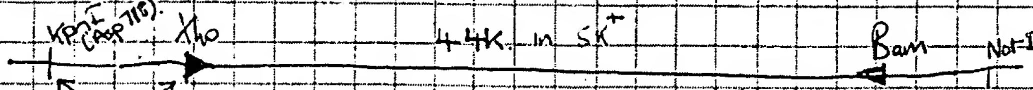
SphI cut 1.5K in pcRII



to be gel purified (actually a doublet).

9 7 4 2

2, 4, 7, and 9 look as though they are correct. Grew up 2 and 4 as O/N Maxi cultures



To Page No.______

Witnessed & Understood by me,	Date	Invented by	Date
Serge W. Kubli [signature]		Recorded by David K. Clarke [signature]	

Project No.______

Book No.______

TITLE Gel analysis of purified 1.3K DNA (Asp/Xho cut).
Hind III digestion of 2.25K maxis 4 + 14.
Gel purification of Sph1.5K and Sph 2.25K in pCII.

From Page No.__

Prepared a 1% agarose gel for 1.3K DNA analysis. Spun down 1.3K DNA rinsed - dried and redissolved in 20μl H_2O. Looked at 1μl on a gel.

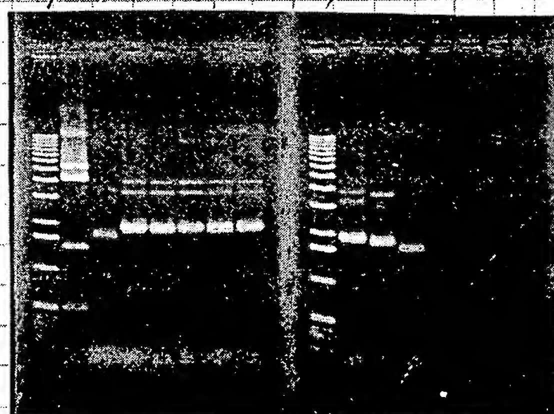
Set up Hind III digestions on 2.25K DNA (2μl DNA) in 20μl - 1μl Enz. ~2hrs 37°C

ran out on a 1% agarose gel to check.

Lisa is doing 4.4K in SK+ 2, and 4. (Maxi preps) - Freezing down 4 and 14 (2.25K in pCII).

1KB ladder

1KB ladder



Gel shows EcoRI digests of Electroporated 4.4K into SK+ vector. None of the clones appear to be correct.

Froze down more hybridomas. Will sub-culture some into 25cm² Flasks.

Spun down maxi-prep 4.4K in SK+ - Looked at 1μl of each on a gel.

Set up EcoRI digests on 1μl to recheck.

To Page No.____

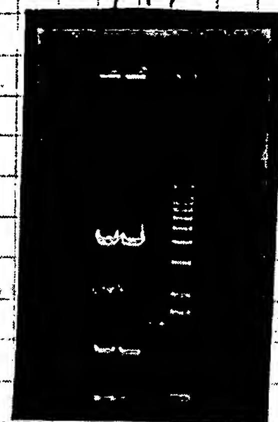
Witnessed & Understood by me, Sage W. Kimble | Date | Invented by | Date

Recorded by David K. Clarke.

Project No.________
TITLE ______________________________ Book No.________

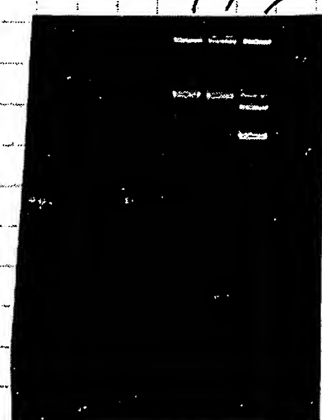
From Page No. 30

14 4 1.3K DNA



Gel shows gel purified 1.3K DNA ~ 50ng µl⁻¹
also shows Hind III digested 2.25K (4 and 14)
They both look fine.

4 } in SK⁺
2 } 4.4K in PCII



gel shows 2 and 4 (4.4K in SK⁺) maxi
preps - which are showing a very low
yield. Will repeat maxis with 7 and 9

Decided to set up O/N Maxis of
7 and 9 to try and get an improved
yield.

To Page No.____

Witnessed & Understood by me, Serge W. Kerbl[illegible] | Date | Invented by | Date

Recorded by David K. Clarke

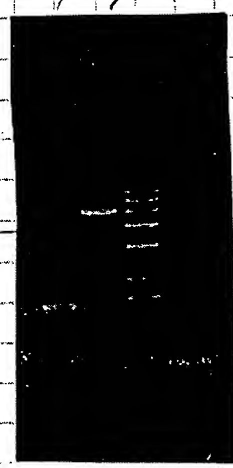
Project No.
Book No.
TITLE Gel analysis of electroeluted 2.25K in pcII and SphI cut 1.5K fragment.

From Page No.

Prepared a 1% agarose gel for analysis of gel purified SphI cut 2.25K in pcII, and SphI fragment of 1.5K DNA. There is ~ 25ng of 1.5K DNA μl^{-1} (half is due to the presence of some vector DNA.

There is ~ 150ng μl^{-1} for 2.25K DNA. – Although there is some contaminating uncut plasmid too.

1.5K DNA.
2.25K in pcII.



uncut 2.25K

Redigested 2.25K with SphI (~1μg) in 30μl with 2μl of enzyme to try and eliminate remaining uncut DNA. – Will then phenol extract – EtOH ppt and phosphatase tomorrow.

Meanwhile prepared L-Agar for restreaking clone banks – poured and dried plates.

Will phosphatase 1μg of 2.25K DNA in 50μl.

8μl 2.25K. 0.5u 2.25 μg
5μl x10
35μl H_2O
2μl phosphatase
50μl – ~30mins at 37°C

added another 1.5μl of phosphatase and left at 37°C for ~40mins longer.

Lisa set up O/N ligation of 1.5K into 2.25K in pcII.

~100ng. 1μl of 2.25K (It was Resuspended in 5μl H_2O)
2μl 1.5K DNA (~ 50ng).
2μl x10 buffer
1μl Enzyme
14μl H_2O
20μl O/N at 37°C

Maxi preps of 4.4K (9 and 7) did not work out.
Decided to colony purify 4.4K (sure) #4

Restreaked entire clone bank

To Page No.

Witnessed & Understood by me. Date
Invented by Date
Recorded by

TITLE Phosphatasing of SphI redigested 2.25K in PCII (Previously cut and gel purified) O/N minis to colony purify 4.4K in SK+

Project No.

Book No.

From Page No.

Checked colony purified 4.4K in SK+ (#4) – there are individual colonies which will be used to set up O/N mini preps.

Set up phosphatase RXN on Redigested (SphI) 2.25K in pcII.
40µl final vol (~1µg of DNA).
2µl phosphatase + 1µl after ~40mins at 37°C.
phenol extracted and etoh ppt'ed with 2µl of glycogen.
(Recessed 5' ends will reduce efficiency.)

Set up 8 O/N mini cultures of potential 4.4K Amp+. Will do minis tomorrow, along with EcoRI digests/gel analysis.

Stored clone bank plates at 4°C – will incubate some for a further 24hrs to improve colony growth.

Lisa carried out transformations with O/N ligation 1.5K + 2.25K in pcII DNA. She also did a vector (unligated) control to check for level of background due to undigested vector (in ligation mixture).

Witnessed & Understood by me. [signature]

Date

Invented by

Date

Recorded by [signature]

To Page No.

Project No.

Book No.

TITLE Mini prep analysis of colony purified 4.4K
Gel analysis of redigested 2.25K, SAP⁺ DNA in PCII.

From Page No.

Checked Lisa's clones 1.5K + 2.25K in PCII - there are many white colonies; however the Controls are pale blue. (may indicate some readthrough of the 2.25K insert).

Carried out mini preps for potential 4.4K clones in SK^{+} vector.

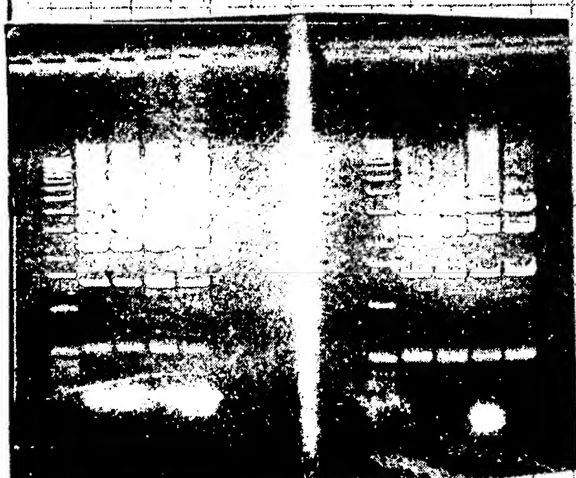
20µl (of 50µl) of the final mini prep suspension in H_2O was digested with EcoRI, and the products were analysed on a 1% agarose gel (The remaining DNA was frozen at -20°c) - May need to use it to transform Late 5?

Spun down redigested (sphI) 2.25K DNA in PCII - resuspended in 10µl H_2O and ran 1µl on a gel to quantitate.

2.25K in PCII SphI cut, SAP^{+}
Redigested

All 8 clones look good - Lisa will grow up ① and ④ as Maxis.

Conc ≡ 100ng µl^{-1}



← Gel shows 8 potential 4.4 clones in pCII - all look fine (they were all derived from original clone 4 (see page 29))

Subcultured hybridomas 46, 47, 48.

To Page No.

Witnessed & Understood by me, [signature]

Date

Invented by

Date

Recorded by [signature]

TITLE: Discussion of progress with Lisa.
Discussion of future direction with Kathy.

Project No.______
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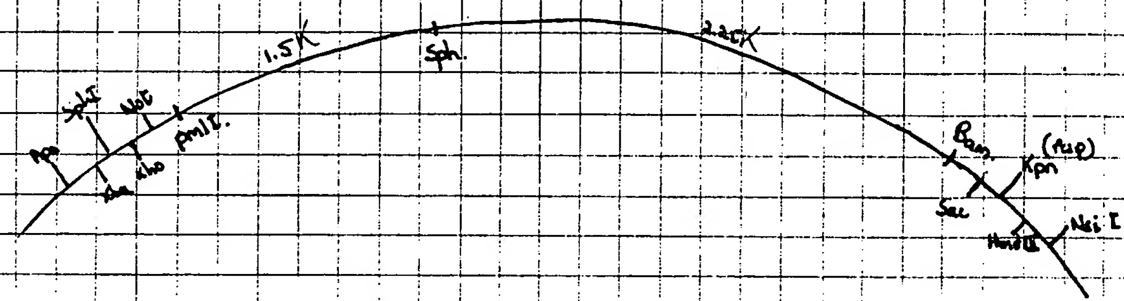
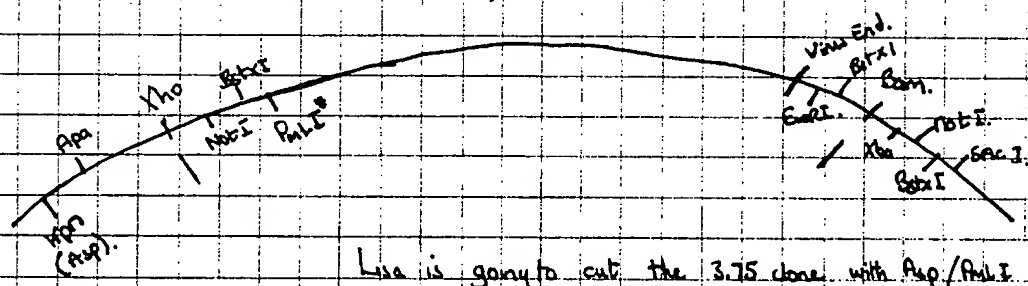
Lisa has done a fine job and has successfully linked the 1.5K and 2.25K clones to give a 3.75K clone in ~~SK+~~. PCII ? pCDNAII



The 3K clone (in PCRII) has been transferred into SK+ (Bam/XhoI site).



Lisa is going to cut the 3.75 clone with Asp/PmlI and gel purify the fragment and ligate it into Asp/PmlI cut (3K in SK+)

Lisa is rechecking the PmlI enzyme (own supply). (the central supply stock did not cut). (The other stock of PmlI is fine)

4.4K DNA could not be grown up in large amounts - will set up 40 mini preps and try to prepare DNA from them.

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Witnessed & Understood by me, Serge W. Kelle | Date | Invented by | Date

Recorded by David K. Clarke